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# STUDIES IN THE METABOLISM OF PLANT CELLS

## XII. IONIC EFFECTS ON OXIDATION OF REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE AND CYTOCHROME $c$ BY PLANT MITOCHONDRIA

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*Summary*

Some effects of ions on the mitochondrial oxidation of reduced diphospho-pyridine nucleotide (DPNH) and cytochrome $c$ were studied. Potassium, sodium, magnesium, calcium, chloride, and orthophosphate consistently increased the rate of DPNH oxidation of beetroot mitochondria which had been isolated in a medium of 0·4M sucrose containing ethylenediaminetetraacetic acid and tris(hydroxymethyl)-aminomethane and washed in sucrose solution; the assay of DPNH oxidation was carried out in a 0·4M sucrose medium. The rate of DPNH oxidation was increased by increasing concentration of chlorides but decreased at higher concentrations. The optimum for divalent chlorides was lower than that for monovalent but the difference was not marked when considered on the basis of ionic strengths. The optimal salt concentrations for stimulation of DPNH oxidation were much lower than those for stimulation of cytochrome $c$ oxidation. It is concluded that this effect of salt on DPNH oxidation by mitochondria could explain salt respiration in plant tissue.

## I. INTRODUCTION

The active and passive roles of mitochondria in the systems responsible for salt uptake by plant cells have been studied by Robertson *et al.* (1955) and Honda and Robertson (1956). The influence of salts on the activity of particulate cyto-chrome oxidase from plants has been investigated by Miller and Evans (1956). These investigations have an important bearing on the problem of salt respiration in intact tissue and are extended in this paper by examination of the influence of salts on the rate of oxidation of both reduced diphosphopyridine nucleotide (DPNH) and cytochrome $c$ by plant mitochondria.

The use of organic acid substrates introduces relatively high concentrations of ions which may mask the stimulating effects of inorganic ions on oxidation and may also induce single-step oxidations which do not necessarily follow the normal electron transport pathway. DPNH has several advantages for study of salt effects: the oxidation of DPNH can be studied in a concentration which introduces negligible quantities of ions in comparison with the ions added for study of salt accumulation, the respiratory system is confined to the electron transport chain by the omission of a dehydrogenase step, and rapid and sensitive methods are available requiring only small quantities of mitochondria.

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Some conditions under which inorganic ions can increase the rate of DPNH oxidation by beetroot mitochondria are reported.

## II. Materials and Methods

Since high yields of mitochondria were not required, modifications of previous methods (Honda and Robertson 1956) were introduced to shorten the preparative time and to decrease the centrifugal force required to sediment the mitochondria isolated from commercial red beetroot (*Beta vulgaris* L.).

Chilled beetroot (100 g) was disintegrated with a Waring Blendor for 20 sec in 110 ml of 0·4M sucrose containing either 0·02M tris(hydroxymethyl)amino-methane (TRIS) or 0·045M TRIS plus 0·005M ethylenediaminetetraacetic acid (EDTA), except where noted. The brei was filtered through muslin and centrifuged for 5 min at 325 $g$. The supernatant was decanted, filtered through muslin, and recentrifuged for 15 min at 10,000 $g$. The resulting supernatant was retained in order to measure its pH, which was taken as the brei pH. The sedimented particles or mitochondria corresponded to those previously used for studies on respiratory activity, active and passive behaviour in salt solutions (Robertson *et al.* 1955; Honda and Robertson 1956), and fine structure of mitochondria (Farrant *et al.* 1956). These mitochondria if used without further manipulations were called "unwashed" mitochondria. "Washed" mitochondria were obtained by resuspending unwashed mitochondria in 7 ml of 0·4M sucrose and sedimenting at 9800 $g$ for 5 min and "twice-washed" mitochondria by repeating this operation. The washed and unwashed mitochondria were resuspended in 7 ml of 0·4M sucrose and placed in a water–ice-bath until portions for nitrogen assay and assay for oxidation were taken. All steps were carried out in a cold room, at less than 5°C, with chilled apparatus or in a refrigerated centrifuge at −1 to 0°C.

The assay system contained 0·10 or 0·20 ml of the mitochondrial suspension, the required amounts of the diluted stock solution of DPNH or reduced cytochrome $c$, sucrose to adjust to the final indicated osmolarity, sufficient TRIS acetate buffer to maintain the required pH, various specified additions, and water to adjust to 3·00 ml final volume. All concentrations were computed as the final concentrations of added constituents. The osmolar concentrations were computed assuming the apparent degrees of dissociation for various salt types were 0·86 for $R^+A^-$, 0·72 for $R^{2+}(A^-)_2$ and $(R^+)_2 A^{2-}$, and 0·45 for $R^{2+}A^{2-}$.*

A model DU Beckman spectrophotometer with photomultiplier attachment was used to measure light absorption of the assay system. DPNH oxidation[†] by the mitochondria was determined by measuring the decrease in optical density at 340 m$\mu$, the absorption peak of DPNH (−$\Delta$O.D.$_{340}$). The reference cuvette contained the assay system without mitochondria and sometimes without DPNH when dilute mitochondrial suspensions were used. All assays were carried out at room temperature. Temperatures and pH values of the assay systems were measured at the conclusion of each experiment. The cytochrome $c$ oxidation rate was determined by measuring the decrease in optical density at 550 m$\mu$.

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* "Handbook of Chemistry and Physics." 33rd Ed. p. 1505, 1951–2.

† That oxidation of DPNH and not decomposition was measured was demonstrated by the fact that change in optical density was prevented by cyanide.

The mitochondrial suspension was added to the rest of the assay system, which was then stirred. Initial optical density readings were taken after about 1·5 min. The interval from initial cutting of the beetroot to the initial optical density reading was usually about 1 hr; depending upon the activity, optical densities of the systems were determined for as long as 30 min. Activities were computed from the slopes of the curves of optical density plotted against time and expressed as change in O.D./unit time/mg N ($-\Delta$O.D.$_{340}$/hr/mg N). The initial slopes were maintained until near the end of the complete oxidation of DPNH. The rapid volume adjustment of the mitochondria to the new osmolar concentration was generally completed within 2 min (cf. Cleland 1952).

The DPNH was a commercial preparation (made by Boehringer & Soehne, Mannheim, Germany) containing 64 per cent. nucleotide, virtually entirely reduced (Dr. K. S. Rowan, personal communication). A stock solution of c. 50 mg per 2 ml was made up containing 1 ml buffer (0.05M TRIS + 0·01M EDTA), sucrose to adjust the osmolarity to 0·4M, and water to 2 ml.* A dilute stock solution was prepared by diluting 0·10 ml of the stock with 3·00 ml of 0·4M sucrose. These stock solutions were stored at freezing temperatures between use.

The cytochrome c was prepared from ox heart by the method of Keilin and Hartree as described by Potter (1951) and dialysed against distilled water to reduce ion contamination to a minimum. The cytochrome c was reduced immediately before use with a slight excess of sodium dithionite and the excess removed with a stream of air.

Stock inorganic ion solutions were prepared either with TRIS or acetate as the balancing ion. TRIS chloride was prepared by neutralizing HCl with TRIS to pH 7·2, and TRIS phosphate by neutralizing orthophosphoric acid with TRIS to pH 7·2. Sodium, potassium, and magnesium were used in the form of acetate salts when differences in cation effects were required. For concentration studies, chlorides of sodium, potassium, calcium, and magnesium were used.

In most experiments mitochondrial nitrogen was determined by difference between the total nitrogen of the suspension and the supernatant from a 5-min centrifugation of the suspension at 14,000 g. In some experiments where the mitochondria had been washed twice, the total nitrogen of the suspension was taken as mitochondrial nitrogen. Total nitrogen was assayed in duplicate by Nesslerization after digestion of the samples with conc. $H_2SO_4$ with $HgSO_4$ as catalyst.

### III. RESULTS

#### (a) Effect of EDTA in Preparation of Mitochondria and of pH in Assay

Figure 1 shows the effects of pH on mitochondria prepared in TRIS alone with orthophosphate and cytochrome c in the assay medium and on mitochondria prepared in TRIS and EDTA. Both preparations showed increasing oxidation rate with increasing pH between 5 and 7 but the rate for mitochondria prepared in TRIS alone decreased between pH 7 and 8. At pH values below 5, an increase

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* The concentrations of DPNH given are uncorrected for impurities. The concentration of EDTA carried over from the stock solution of DPNH to the assay system was negligible compared with the concentrations of ions added.

in the optical density of mitochondrial suspensions was induced for periods as long as 5 min after the addition of mitochondria to the assay system. Thereafter, the optical density decreased as might be expected for a slow oxidation of DPNH. In view of these results, all subsequent experiments were carried out between pH 7 and 8, both in isolating the mitochondria and in assay.
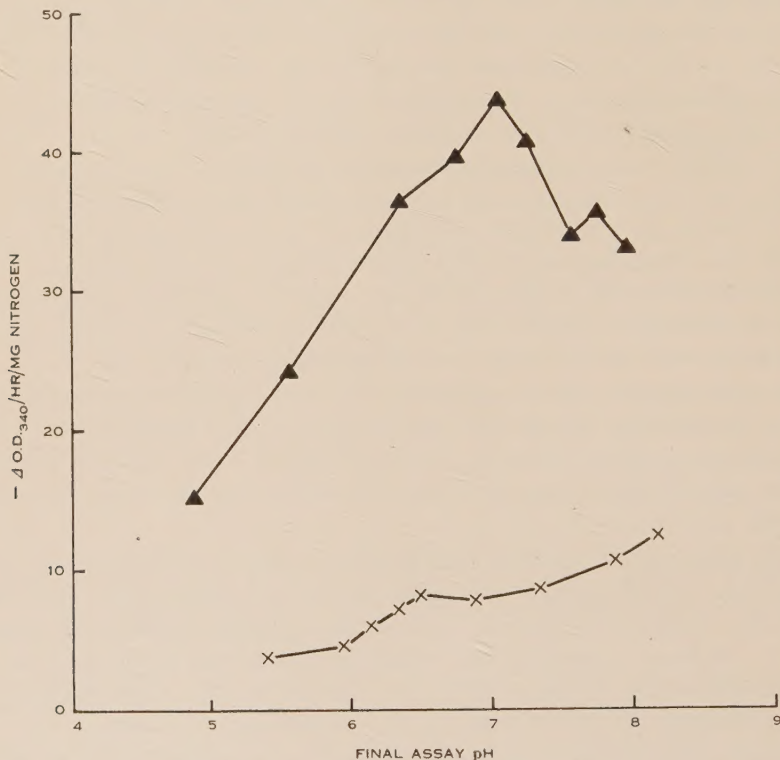


Fig. 1.—Effect of pH adjusted with TRIS acetate buffer on DPNH oxidation. ▲ Mitochondria isolated in TRIS, homogenate pH 7·7, washed in sucrose. Assay details: mitochondrial nitrogen, 0·030 mg; DPNH, 55·6 μg per ml; orthophosphate, 6·3 mM; cytochrome c, 13·5 μM; temperature 22°C, sucrose concn. 0·38M, and osmolar concn. 0·4M. ✕ Mitochondria isolated in TRIS plus EDTA, homogenate pH 7·9, washed in sucrose. Assay details: mitochondrial nitrogen, 0·016 mg; DPNH, 38·7 μg per ml; sucrose concn. 0·4M; osmolar concn. 0·41M.

## (b) Effect of Tonicity

To test the effects of tonicity, mitochondria were isolated in 0·4M sucrose plus additions and diluted in assay solutions of various osmolar concentrations. The activity of washed mitochondria prepared in TRIS and EDTA remained low at all osmolar concentrations (Fig. 2). The rate of DPNH oxidation of washed mitochondria prepared in TRIS alone was greater than that of washed mitochondria prepared in TRIS and EDTA but decreased as the tonicity was lowered below 0·22 osmolar; orthophosphate and cytochrome c added to washed mitochondria prepared in TRIS increased the rate of oxidation. Unwashed mitochondria of both

types of preparation with orthophosphate and cytochrome $c$ added showed a marked increase in the rate of DPNH oxidation as the tonicity fell below 0·22 osmolar.
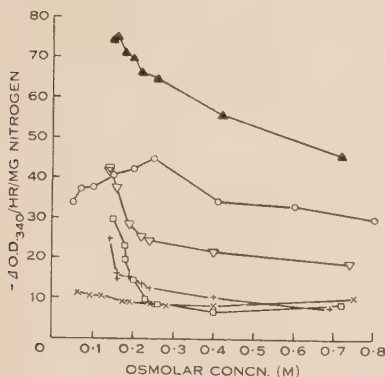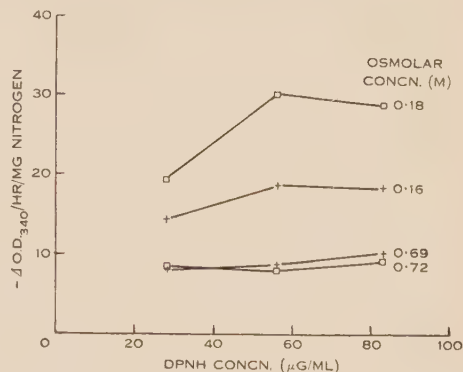


Fig. 2                    Fig. 3

Fig. 2.—Effect of osmolar concentration on DPNH oxidation. ▲ Mitochondria isolated in TRIS, homogenate pH 7·9, washed in sucrose. Assay details: mitochondrial nitrogen, 0·013 mg; DPNH, 51·6 $\mu$g per ml; orthophosphate, 22·8 $\mu$M; cytochrome $c$, 0·9 $\mu$M; TRIS, 3·1 × $10^{-2}$M; acetate, 3·5 × $10^{-2}$M; pH 7·2; temperature 22°C; non-sucrose osmolar contribution, 0·11M. ○ Mitochondria isolated in TRIS, washed in sucrose. Assay details: mitochondrial nitrogen, 0·014 mg; DPNH, 62·3 $\mu$g per ml; TRIS, 3·1 × $10^{-3}$M; acetate, 3·5 × $10^{-3}$M; pH 7·4; temperature 19°C; non-sucrose osmolar contribution, 0·01M. ▽ Unwashed mitochondria isolated in TRIS, homogenate pH 7·7. Assay details: mitochondrial nitrogen, 0·010–0·013 mg; DPNH, 27·8 $\mu$g per ml; orthophosphate, 6·3 mM; cytochrome $c$, 6·4 $\mu$M; TRIS, 6·0 × $10^{-2}$M; acetate, 4·6 × $10^{-2}$M; pH 7·0–7·5; temperature 24°C; non-sucrose osmolar contribution 0·11M. □ Unwashed mitochondria isolated in TRIS, homogenate pH 8·1–8·3. Assay details: mitochondrial nitrogen, 0·027–0·028 mg; DPNH, 27·8 $\mu$g per ml; orthophosphate, 6·3 mM; TRIS, 6·0 × $10^{-2}$M; acetate, 4·6 × $10^{-2}$M; pH 7·9; temperature, 25°C; non-sucrose osmolar contribution 0·11M. + Unwashed mitochondria isolated in TRIS plus EDTA, homogenate pH 8·1. Assay details: mitochondrial nitrogen, 0·013–0·015 mg; DPNH, 27·8 $\mu$g per ml; orthophosphate, 6·3 mM; cytochrome $c$, 6·4 $\mu$M; TRIS, 6·0 × $10^{-2}$M; acetate, 4·6 × $10^{-2}$M; pH 7·9–8·1; temperature 24°C; non-sucrose osmolar contribution, 0·1M. ✕ Mitochondria isolated in TRIS plus EDTA, homogenate pH 8·0, washed in sucrose. Assay details: mitochondrial nitrogen, 0·014 mg; DPNH, 51·6 $\mu$g per ml; TRIS, 3·1 × $10^{-2}$M; acetate, 3·5 × $10^{-2}$M; pH 7·2; temperature 20°C; non-sucrose osmolar contribution, 0·01M.

Fig. 3.—Effects of DPNH concn. and osmolar concn. on oxidation by unwashed mitochondria. □ Mitochondria isolated in TRIS, homogenate pH 8·3. Assay details: mitochondrial nitrogen, 0·028 mg; orthophosphate, 6·3 mM; TRIS, 6·0 × $10^{-2}$M; acetate, 4·6 × $10^{-2}$M; pH 7·9; temperature 25°C; non-sucrose osmolar contribution, 0·11M. + Mitochondria isolated in TRIS plus EDTA, homogenate pH 8·1. Assay details: mitochondrial nitrogen, 0·015 mg; orthophosphate, 6·3 mM; cytochrome $c$, 6·4 $\mu$M; TRIS, 6·0 × $10^{-2}$M; acetate, 4·6 × $10^{-2}$M; pH 7·9; temperature 24°C; non-sucrose osmolar contribution, 0·1M.

In high osmolar solutions, presumably hypertonic, the rate of DPNH oxidation by mitochondria was low and largely independent of DPNH concentrations (Fig. 3). In hypotonic solutions, 0·18 and 0·16 osmolar, the mitochondria oxidized DPNH more rapidly and the rate appeared to increase with increasing DPNH concentration until a saturating concentration was again reached.

In view of these results subsequent experiments on the effects of ions were carried out with the osmolarity adjusted to about 0·4 or above.

### (c) Effect of Cytochrome c on DPNH Oxidation Rate

Figure 4 shows that the rate of DPNH oxidation by mitochondria was increased up to 2·5 times by the addition of cytochrome c. The saturation con-
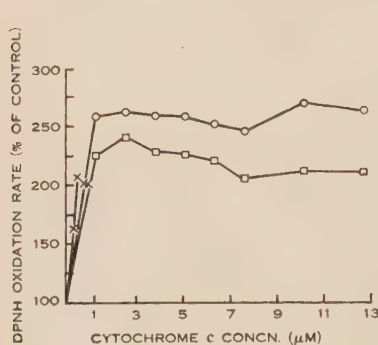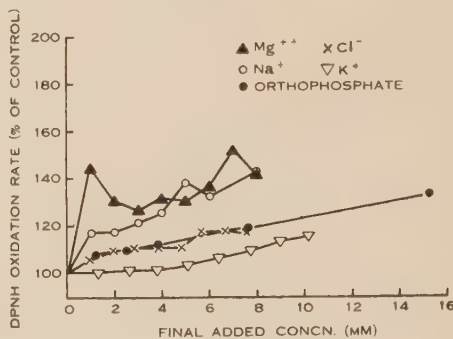


Fig. 4.                                    Fig. 5

Fig. 4.—Effect of cytochrome c concn. on DPNH oxidation by unwashed mitochondria. □ Isolated in 0·02M TRIS, homogenate pH 8·0. Assay details: mitochondrial nitrogen, 0·015 mg; DPNH, 55·6 μg/ml plus EDTA, $3·2 \times 10^{-4}$M; orthophosphate, 6·2 mM; TRIS, $6·0 \times 10^{-2}$M; acetate, $4·6 \times 10^{-2}$M; sucrose, 0·29M; osmolar, 0·4M; pH 7·9; temperature 24°C; control rate, $-\Delta$O.D./hr/mg N, 10·5. ○ Isolated in 0·07M TRIS plus 0·01M EDTA, homogenate pH 8·5. Assay details: mitochondrial nitrogen, 0·018 mg; DPNH, 55·6 μg/ml plus EDTA, $3·2 \times 10^{-4}$M; orthophosphate, 6·2 mM; TRIS, $6·0 \times 10^{-2}$M; acetate, $4·6 \times 10^{-2}$M; sucrose, 0·29M; osmolar, 0·4M; pH 7·9; temperature 25°C; control rate, $-\Delta$O.D./hr/mg N, 6·6. ✕ Isolated in 0·045M TRIS plus 0·005M EDTA, homogenate pH 7·8. Assay details: mitochondrial nitrogen, 0·030 mg; DPNH 38·7 μg/ml plus EDTA $8·0 \times 10^{-5}$M; TRIS, $3·1 \times 10^{-3}$M; acetate, $3·5 \times 10^{-3}$M; sucrose, 0·39M; osmolar, 0·4M; pH 7·1; temperature 18°C; control rate, $-\Delta$O.D./hr/mg N, 10·4.

Fig. 5.—Effect of inorganic cation and anion concentrations on DPNH oxidation by washed mitochondria isolated in 0·045M TRIS plus 0·005M EDTA. Assay details: DPNH, 62·3 μg/ml; EDTA, $1·1 \times 10^{-4}$M; TRIS, $3·1 \times 10^{-3}$M; acetate, $3·5 \times 10^{-3}$M; sucrose, 0·4M; osmolar, 0·41M. ▲ $Mg^{++}$, homogenate pH 7·7. Assay details: mitochondrial nitrogen, 0·015 mg; pH 7·3; temperature 20°C; control rate, $-\Delta$O.D./hr/mg N, 6·1. ○ $Na^+$, homogenate pH 7·6. Assay details: mitochondrial nitrogen, 0·007 mg; pH 7·2; temperature 20°C; control rate $-\Delta$O.D./hr/mg N, 23·5. ● Orthophosphate, homogenate pH 7·6. Assay details: mitochondrial nitrogen, 0·007 mg; pH 7·6; temperature 19°C; control rate, $-\Delta$O.D./hr/mg N, 19·2. ✕ $Cl^-$, homogenate pH 8·0. Assay details: mitochondrial nitrogen, 0·006 mg; pH 7·5; temperature 19°C; control rate $-\Delta$O.D./hr/mg N, 27·1. ▽ $K^+$, homogenate pH 7·6. Assay details: mitochondrial nitrogen, 0·014 mg; pH 7·2; temp. 20°C; control rate, $-\Delta$O.D./hr/mg N, 83·5.

centration of added cytochrome c was below $10^{-6}$M for mitochondria prepared in both TRIS and TRIS plus EDTA.

### (d) Effect of Ions on DPNH Oxidation Rate

Under certain conditions the addition of different inorganic cations and anions was found to increase the rate of DPNH oxidation by washed mitochondria. Un-

washed mitochondria showed an increased rate of DPNH oxidation with cyto-chrome $c$ but usually not with increasing ionic concentrations of $Na^+$, $K^+$, $Mg^{++}$, $Cl^-$, or orthophosphate.

Four types of washed mitochondrial preparations were examined for salt effects:

(i) Mitochondria prepared in TRIS and assayed at constant osmolar concentration.

(ii) Mitochondria prepared in TRIS and assayed at constant sucrose concentration but increasing osmolar concentration.

(iii) Mitochondria prepared in TRIS and EDTA and assayed at constant osmolar concentration.

(iv) Mitochondria prepared in TRIS and EDTA and assayed at constant sucrose concentration.

Figure 5 shows representative experiments on effects of ions at low concentrations upon DPNH oxidation by washed mitochondria prepared in TRIS and EDTA and assayed at constant sucrose concentration. Increased rates of DPNH

TABLE 1

COMBINED EFFECTS OF SOME IONS, ATP, AND CYTOCHROME $c$ ON DPNH OXIDATION BY WASHED MITOCHONDRIA, ISOLATED IN 0·045M TRIS + 0·005M EDTA + 0·4M SUCROSE, ASSAYED UNDER CONSTANT OSMOLAR CONCN.

Assay details for control: mitochondrial nitrogen, 0·0095 mg; DPNH, 38·7 $\mu$g/ml; EDTA, 8·0 $\times$ $10^{-5}$M; sucrose, 0·39M; osmolarity, 0·4M; TRIS, 3·1 $\times$ $10^{-3}$M; acetate, 3·5 $\times$ $10^{-3}$M; pH 7·0; temperature 20°C; rate, $-\Delta$O.D./hr/mg N, 18·8. Assay details for complete medium: as in the control with $Mg^{++}$, 1·3 mM; orthophosphate, 2·5 mM; ATP (sodium salt), 1·1 mM; TRIS chloride, 8·4 mM; cytochrome $c$, 0·4 $\mu$M

| Treatment | DPNH Oxidation Rate as Per Cent. of Control |
|---|---|
| Control | 100 |
| Control + TRIS chloride, 8·4 mM | 162 |
| Control + TRIS chloride + cytochrome $c$, 0·4 $\mu$M | 240 |
| Complete medium | 259 |
| Complete medium, less $Mg^{++}$ | 257 |
| Complete medium, less orthophosphate | 264 |
| Complete medium, less ATP (sodium salt) | 259 |
| Complete medium, less TRIS chloride | 264 |
| Complete medium, less cytochrome $c$ | 206 |

oxidation with the additions of $Mg^{++}$ and $Na^+$ as acetates and with TRIS ortho-phosphate were found for all types of washed mitochondrial preparations. $K^+$ added as acetate had little or no effect on the rate of DPNH oxidation with the exception of mitochondria prepared in TRIS and EDTA. Demonstration of

chloride effects was obtained consistently only with washed mitochondria prepared in TRIS and EDTA under conditions of constant sucrose concentration and for brief transient times for washed mitochondria prepared in TRIS. The sodium salt of adenosine triphosphate (ATP) neither increased nor decreased the oxidation rate of DPNH by any type of mitochondrial preparation.

Table 1 shows that withdrawal of one ion from a mixture of several ions with cytochrome $c$ did not decrease the maximal DPNH oxidation rate by washed mitochondria prepared in TRIS and EDTA. Only the withdrawal of cytochrome $c$

TABLE 2

COMBINED EFFECTS OF SOME IONS AND CYTOCHROME $c$ ON DPNH OXIDATION BY WASHED MITOCHONDRIA ISOLATED IN $0 \cdot 02$M TRIS AND ASSAYED UNDER CONSTANT OSMOLAR CONCENTRATION

Assay details for control: mitochondrial nitrogen, $0 \cdot 0098$ mg; DPNH, $62 \cdot 3$ $\mu$g/ml; EDTA, $1 \cdot 1 \times 10^{-4}$M; sucrose, $0 \cdot 4$M; osmolar, $0 \cdot 4$M; TRIS, $3 \cdot 1 \times 10^{-3}$M; acetate, $3 \cdot 5 \times 10^{-3}$M; pH $7 \cdot 3$; temperature 20°C; rate, $-\Delta$O.D./hr/mg N, $32 \cdot 3$. Assay details for complete medium: as in the control with $Mg^{++}$, 5 mM; orthophosphate, $7 \cdot 6$ mM; ATP (sodium salt), $1 \cdot 1$ mM; TRIS chloride, $8 \cdot 4$ mM; cytochrome $c$, $0 \cdot 4$ $\mu$M

| Treatment | DPNH Oxidation Rate as Per Cent. of Control |
|---|---|
| Control | 100 |
| Control + TRIS chloride, $7 \cdot 5$ mM | 116 |
| Control + TRIS chloride, $7 \cdot 5$ mM + cytochrome $c$, $0 \cdot 2$ $\mu$M | 236 |
| Control + cytochrome $c$, $0 \cdot 2$ $\mu$M | 193 |
| Control + $Na^+$, $7 \cdot 0$ mM | 120 |
| Control + $Na^+$ ($7 \cdot 0$ mM) + $Cl^-$ ($7 \cdot 5$ mM) | 125 |
| Complete medium | 215 |
| Complete medium, less $Mg^{++}$ | 211 |
| Complete medium, less $Mg^{++}$, less orthophosphate | 252 |

decreased the rate from the maximal and only chloride plus cytochrome $c$ was required to establish the maximal rate. Table 2 also shows that, even for washed mitochondria prepared in TRIS, cytochrome $c$ plus chloride alone was sufficient to establish the maximal rate of DPNH oxidation although chloride itself only slightly increased the rate.

Of the other ions, when added with cytochrome $c$, only $Na^+$ was as effective as chloride in establishing the maximal rate of DPNH oxidation by washed mitochondria. When chloride alone had little effect and chloride plus cytochrome $c$ did not establish maximal oxidation rates, the addition of $Na^+$, $Mg^{++}$, or orthophosphate, but not $K^+$, gave maximal oxidation rates. When three or more ions were added with cytochrome $c$ to washed mitochondria, the omission of any one did not depress the rate of DPNH oxidation.

These results suggest that endogenous salts were sufficient to maintain maximal rate of DPNH oxidation by unwashed mitochondria, so added salts had no additional effect. The endogenous salt could be removed by washing, but ion effects were found consistently in washed mitochondria only when EDTA was added to the isolation medium prior to washing. This might suggest that EDTA removed, from the mitochondria, cations normally required to stimulate the oxidation; this would be consistent with our observation that effects of cations added to such mitochondria were easy to demonstrate. Since the withdrawal of any one anion or cation from a mixture of three or more ions did not depress the induced rapid rate of DPNH oxidation, specificity of the ions was not established.
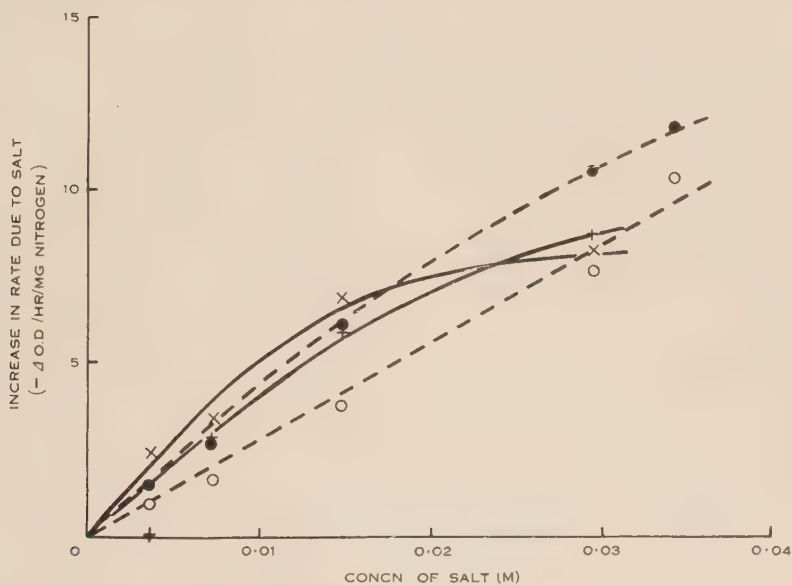


Fig. 6.—Effect of increasing concentration of KCl and NaCl on DPNH oxidation by twice-washed mitochondria isolated in 0·045M TRIS plus 0·005M EDTA plus 0·4M sucrose. Assay details: DPNH 64·5 $\mu$g/ml plus EDTA 1·7 × $10^{-4}$M; cytochrome $c$, 2 $\mu$M; TRIS, 3·1 × $10^{-3}$M; acetate 3·5 × $10^{-3}$M; sucrose, 0·4M. X KCl effect; homogenate pH 7·9. Assay details: mitochondrial nitrogen, 0·031 mg; pH 7·3–7·2. + KCl effect; homogenate pH 8·2. Assay details: mitochondrial nitrogen, 0·022 mg; pH 7·3–7·2. ○ NaCl effect; homogenate pH 7·9. Assay details: mitochondrial nitrogen, 0·016 mg; pH 7·2–7·1. ● NaCl effect; homogenate pH 8·1. Assay details: mitochondrial nitrogen, 0·014 mg; pH 7·5–7·2.

### (e) Effect of Concentration

The effects of concentration of chlorides on DPNH oxidation were examined with twice-washed mitochondria in 0·4M sucrose solution (Figs. 6 and 7). In such mitochondria, a basal rate of oxidation in the absence of salt was always obtained and varied from preparation to preparation. Increase in concentration of chlorides increased the rate of oxidation at lower concentrations but decreased the rate at higher concentrations. No marked differences were observed between sodium and potassium chlorides but calcium chloride showed a peak in the stimulated respiration at lower normality than the monovalent ions. Results of experiments over a narrow concentration range are shown in Figure 6.

### ( f ) *Comparison of DPNH Oxidation and Cytochrome* c *Oxidation*

The possibility that the effect of salt was entirely due to its action on the cytochrome oxidase system was examined by comparing DPNH oxidation with cytochrome $c$ oxidation on replicate preparations (Fig. 7). Three different preparations were used and, since some variation was to be expected, the effect of potassium chloride on both DPNH and cytochrome $c$ oxidation is given in each preparation. The differences between the curves for potassium chloride effects are only small and sodium chloride and potassium chloride resemble each other (Fig. 7(a)). The divalent salts, calcium and magnesium chlorides, are different from monovalent salts in their effects on both DPNH oxidation and cytochrome $c$ oxidation (Figs. 7(b) and 7(c)). The effects of these chlorides on oxidation rate of cytochrome $c$ are very
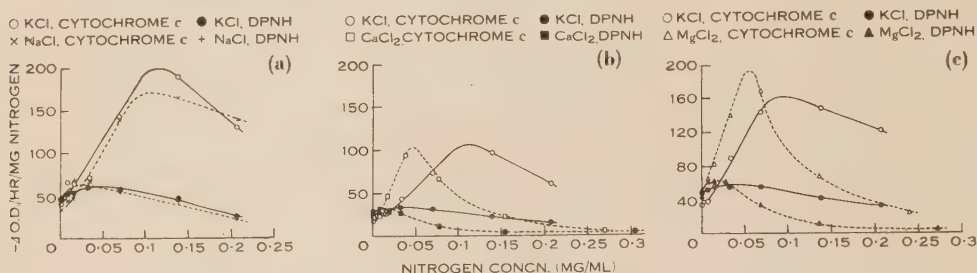


Fig. 7.—Effects of sodium, potassium, magnesium, and calcium chlorides on DPNH and cytochrome $c$ oxidation by twice-washed mitochondria isolated in 0·045M TRIS plus 0·005M EDTA plus 0·4M sucrose. Assay details: DPNH oxidation: DPNH, 64·5 $\mu$g/ml; EDTA, $1·7 \times 10^{-4}$M; cytochrome $c$, 2 $\mu$M; TRIS, $3·1 \times 10^{-3}$M; acetate, $3·5 \times 10^{-3}$M; sucrose 0·4M. Cytochrome $c$ oxidation: reduced cytochrome $c$, 41·33 $\mu$M; TRIS, $3·1 \times 10^{-3}$M; acetate, $3·5 \times 10^{-3}$M; sucrose 0·4M. (a) KCl and NaCl effect: mitochondrial nitrogen, 0·049 mg. (b) KCl and CaCl$_2$ effect: mitochondrial nitrogen, 0·087 mg. (c) KCl and MgCl$_2$ effect: mitochondrial nitrogen, 0·053 mg.

similar to those obtained by Miller and Evans (1956) with plant mitochondria from four other sources. The cytochrome $c$ results differ from the DPNH results, however. Generally speaking, increase in salt concentration stimulates DPNH oxidation to its maximum at a lower concentration than the maximum for cytochrome $c$ oxidation. Subsequently, there is a slow fall with increasing concentrations. The divalent chlorides produce a peak in both oxidations at lower normalities than the monovalent salts but this difference is much less when the results are plotted against ionic strength (Fig. 8). At higher ionic strength, the divalent salts cause a decrease in the rate of oxidation which is more marked than that caused by the monovalent salts.

These results show that, while both cytochrome oxidation and DPNH oxidation are stimulated by the presence of ions *in vitro*, the DPNH oxidation is more sensitive to depressant effects at higher concentrations.

### IV. Discussion

Some characteristics of DPNH oxidation by lupine mitochondria have been reported (Humphreys and Conn 1956). The isolation and assay methods which

differed from those used for our beetroot mitochondria may account for some difference in DPNH oxidation by lupine mitochondria. For beetroot mitochondria, saturation levels of cytochrome $c$ enhancing the rate of DPNH oxidation were about 10–20 times lower than for lupine mitochondria. Without added cytochrome $c$ the rates of DPNH oxidation were generally two to three times greater for lupine mitochondria than beetroot mitochondria. These greater activities were obtained in solutions of low tonicity, probably hypotonic, where greater activities may be
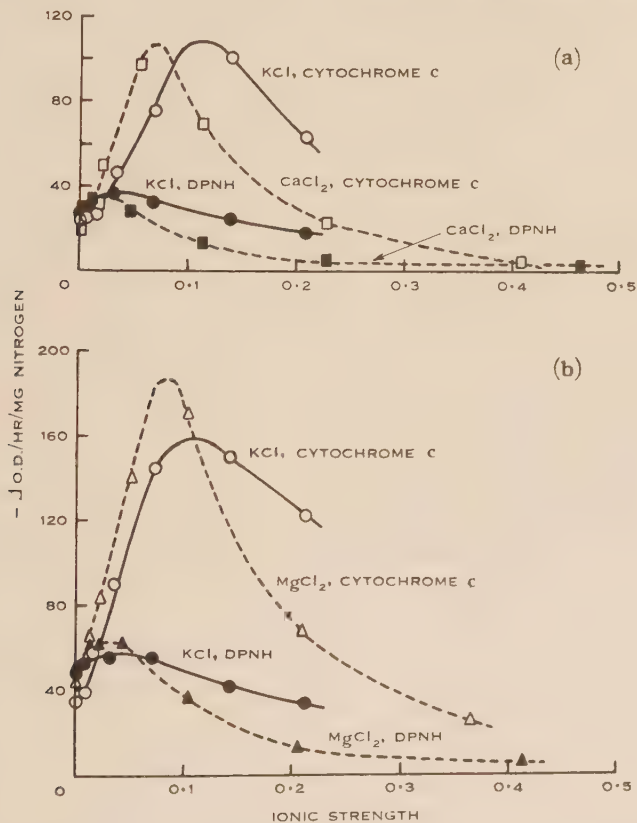


Fig. 8.—Effects of sodium, potassium, magnesium, and calcium chlorides on DPNH and cytochrome $c$ oxidation plotted against ionic strength; same data as in Figure 7. (a) KCl and CaCl₂ effect. (b) KCl and MgCl₂ effect.

expected compared with those of beetroot mitochondria in hypertonic solutions. The decreased rate of DPNH oxidation by lupine mitochondria in solutions of low sucrose concentration compared with that in high sucrose concentration, in the absence of added cytochrome $c$, recalls the behaviour of washed beetroot mitochondria prepared in TRIS and assayed without added cytochrome $c$. The addition of cytochrome $c$ to both lupine and beetroot mitochondria permitted the demonstration of high rates of DPNH oxidation in hypotonic solutions compared with hypertonic solutions. This could be due to replacing endogenous cytochrome $c$

which had been removed during the preparation, an effect which in beetroot mito-
chondria can be duplicated by omission of washing the mitochondria, and, in part,
pretreatment with EDTA during the isolation procedure. It could also be due to a
non-specific effect of the cytochrome $c$ protein, since protein among other things
can stabilize the activity of succinoxidase (cf. Keilin and Hartree 1947).

The use of DPNH as a respiratory substrate for study of salt respiration of
mitochondria raises the question of associating physiological behaviour of intact
mitochondria with the oxidation of exogenous DPNH. Liver mitochondria effectively
carried out phosphorylation coupled with the oxidation of exogenous DPNH
(Lehninger 1951) only if subjected to extreme hypotonicity. It was suggested that
mitochondrial permeability to DPNH was increased, or the mitochondrial structure
altered by the incubation in distilled water. Our results with beet mitochondria at
different tonicities (Figs. 2 and 3) could also have been due to more sites for DPNH
oxidation becoming available in hypotonic solutions. If, for instance, the particle
in hypertonic or isotonic solutions was highly impermeable to exogenous DPNH,
then oxidation would take place only at sites on the surface and increasing concen-
tration of DPNH would have little effect (Fig. 3). When the particle swelled in
hypotonic solutions, the permeability might increase, more internal sites for DPNH
oxidation become available, and the rate of oxidation be limited by diffusion of
exogenous DPNH to these sites. If little change in membrane permeability occurred
in the hypertonic to isotonic range, the same activity would be expected at all
concentrations as is suggested by Figure 2. At hypotonic concentration, however,
the increased swelling may result in increased accessibility of the internal sites,
unless too much disorganization occurs which may have happened with the TRIS-
washed mitochondria which showed decreased rate of DPNH oxidation at lower
concentrations. It is, of course, possible that EDTA inhibits the activity of the
internal sites at low osmolar concentrations and that this explains the difference
between the mitochondria prepared in TRIS and those prepared in TRIS plus
EDTA. Cytochrome $c$ and orthophosphate and the omission of the washing appeared
to stabilize the internal sites.

The ion effects which increased the rate of DPNH oxidation occurred con-
sistently only with mitochondria which were apparently intact. For example, the
addition of chloride increased the rate of DPNH oxidation most consistently for
mitochondria isolated in EDTA solutions. EDTA was found to prevent the increase
in permeability of rat-heart sarcosomes (Cleland 1952), part of which effect may be
attributed to protection by chelation of calcium (Slater and Cleland 1952). Calcium
may activate ATPase (Potter, Siekevitz, and Simonson 1953), and lowered endo-
genous ATP content was correlated with the swelling of mitochondria (Brenner-
Holzach and Raaflaub 1954). Both ATP and EDTA may complex calcium within
the mitochondria (Raaflaub 1955). Isolation of beetroot mitochondria in EDTA
solutions was found to decrease the content of mitochondrial calcium (Honda and
Robertson 1956) and to increase both the rate of oxidations of succinate, malate,
and α-ketoglutarate and the accompanying phosphorylations (Robertson and Tobin,
unpublished data). Although previous experiments did not show beneficial effects
of EDTA in the demonstration of salt accumulation by mitochondria (Honda and

Robertson 1956) or improvement in the resolution of their fine structure (Farrant *et al.* 1956), the modifications in preparative methods have now shown effects on mitochondrial oxidation of DPNH.

The ion effects which increased the rate of DPNH oxidation cannot be attributed to the DPNH oxidation step alone but may act on other steps in the mitochondrial respiratory chain. The increased rate of oxidation could, for instance, be due to the ions stimulating the cytochrome *c*–cytochrome oxidase steps, if these steps had been rate limiting. The fact that the further increase in concentration reduces the rate of DPNH oxidation while still stimulating cytochrome *c* oxidase suggests, however, that cytochrome *c* oxidation is not the rate-determining step in DPNH oxidation at these ionic concentrations. It is possible for salt to affect, directly or indirectly, the electron transport chain in several different ways and at the present stage of our knowledge, speculation on how it is affected would seem premature.

When no salt was added to the twice-washed mitochondria, the rate of DPNH oxidation was about eight times that of added cytochrome *c* oxidation (in terms of electron transport), assuming $\epsilon_{340\,m\mu}$ DPNH $= 6 \cdot 22 \times 10^6$ cm$^2$/mole and $\Delta\epsilon_{550\,m\mu}$ cytochrome $c = 1 \cdot 96 \times 10^7$ cm$^2$ /mole. In the presence of added salt the maximum rate of DPNH oxidation was about twice the maximum rate of added cytochrome *c* oxidation. This observation, taking into consideration the low concentration of cytochrome *c* necessary to give maximal rate of DPNH oxidation, suggests that the endogenous cytochrome *c*, together with the small amount added to replace what had been removed from the particles during preparation, is efficient in the electron transport from DPNH to oxygen. This system is more efficient than that in which reduced cytochrome *c* is supplied, probably because DPNH molecules can reach the sites of their oxidation more readily than reduced cytochrome *c* molecules. Alternatively, centres of oxidation of cytochrome *c* may be blocked by oxidized cytochrome *c* already occupying these centres. The ratio of the DPNH oxidation to cytochrome *c* oxidation changes at higher concentrations of salts, when the DPNH oxidation becomes depressed relatively more than the cytochrome oxidation.

While there is evidence for some differences in the effects of different ions supplied, there is no evidence in these experiments for the suggestion that the stimulation is an anion effect as originally proposed by Lundegårdh (1940). It appears that the stimulation is due to the presence of free ions and that cations are important. These results, like those of Miller and Evans (1956), may have some bearing on the explanation of the effects of salts on respiration in intact tissue. Miller and Evans rightly point out that the concentration of monovalent ions (0·1M) required for maximum oxidation of cytochrome *c* is considerably higher than the concentration of salts (approx. 0·005–0·01M) necessary for maximum salt respiration in tissue. Our results show that the optimum stimulation of DPNH oxidation occurs at about 0·01M and that of cytochrome oxidation at about 0·15M. Our results, taken with those of Miller and Evans, suggest that the DPNH oxidation is more sensitive to salt than the cytochrome oxidation and reaches its maximum at an external concentration similar to that giving maximal stimulation of respiration in carrot tissue (Robertson and Wilkins 1948). It is possible that the mitochondria of intact tissue

may be even more sensitive in their response to free ions than mitochondria *in vitro.*

The salt respiration is observed only in tissue which has been treated (e.g. washing for approved periods in distilled water) in such a way as to reduce the concentration of ions in the external medium and in the free space, including the cytoplasm of the tissue. The fact that leakage of free ions from such aerated tissue is negligible suggests that the free ion concentration of the free space in the cytoplasm is very low and that the ions in contact with the surfaces of the mitochondria are present in negligible amounts. Under such conditions, the salt respiration would be negligible. When salt is applied to the external solution, some ions diffuse into the free space and mobile ions come into contact with the mitochondria. If they exert a stimulatory affect similar to that observed in extracted mitochondria, salt respiration would result.

## V. Acknowledgments

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# THE PHYSIOLOGY OF SUGAR-CANE

## I. STUDIES ON THE NUTRITIONAL AND PHYSIOLOGICAL INTERRELATIONSHIPS OF THE GERMINATING CUTTING

### By K. T. GLASZIOU*

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*Summary*

Nutritional and physiological relationships in germinating cuttings of sugar-cane have been studied under conditions where all nutritional requirements of the growing regions were obtained from the cutting.

The changes in moisture content, fresh weight, and dry weight which occurred in various morphological regions are discussed, and details are also given of changes in the carbohydrate content, in the nitrogenous fractions, and the phosphorus fractions.

The dry weight of the new shoot increased in an approximately linear manner over a 4-week period, indicating that growth was not limited by a diminishing supply of metabolites moving from the cutting. Extrapolation of the curves for sugar contents showed that the concentrations in the shoot and the cutting would have been the same at about 40 days from planting.

The results indicated that the transport of sugars from the cutting was limited by a first-order chemical reaction, and not by simple diffusion. Water movement and hormonal relationships during germination and growth have been discussed.

## I. INTRODUCTION

The germination (axillary bud growth) of sugar-cane sets and the subsequent growth of the new shoot has been the subject of considerable physiological interest, and is of great economic importance. Sugar-cane is normally propagated by cuttings from the stalks of mature plants. Axillary buds are located at the nodes of the stalks, and immediately above are the root bands which contain a large number of root primordia. Germination of the set consists of the growth and development of the bud to give a new shoot complete with its own leaves, roots, etc., and in addition the development of the root primordia in the root band to give the set roots.

Went (1896) has made the only extensive study of food metabolism in germinating cuttings, and has suggested that the developing bud exerted an influence on the cutting resulting in a conversion of sucrose to glucose which was then stored around the bundles in the form of starch to be transported to the growing regions as required. The starch decreased after 10 days, and had disappeared after 20 days. As sugar flowed from the set to the germinating bud, it was partly stored as starch, particularly at the growing point. More recently, Sen (1954) found that the sucrose content of germinated sets gradually decreased to zero over a 3-month period, while

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the glucose content rose for about 6 weeks and then decreased. Sen studied the effect of increasing the carbohydrate content of sets by soaking in molasses, and concluded that a high sugar content in sets gave better juice quality at harvest.

The effects of application of fertilizers at planting was studied by Arceneaux (1948) who reported that heavy dressings of nitrogen increased the germination rate by about 25 per cent. Evans (1938) placed commercial fertilizers inside cuttings and obtained better development of young plants. A number of other studies on the effect of the length of the cutting on germination and growth have been reviewed by van Dillewijn (1952). In general, the larger the cutting attached to the bud the better bud development will be.

The effect of growth-regulating substances on germination has been studied extensively and much of the work has been reviewed by van Dillewijn (1952). Placing cuttings in a vertical position favours bud development while a horizontal position favours root development (van Dillewijn 1948). The application of 3-indolylacetic acid (IAA) to the upper cut surface results in a stimulation of root germination and inhibition of lateral bud development. Khan and Hall (1954) found that a combination of IAA and sucrose increased germination, root weight, and root volume. Cuttings from the upper stalk were more responsive to added sucrose and those from the lower stalk to applied IAA, which suggested the necessity of a suitable auxin–sugar balance for optimum response. Pre-exposure to ethylene was antagonistic to subsequent auxin treatment in terms of rooting, a result which is complementary to that of van Overbeek et al. (1944), quoted by van Dillewijn (1952), who found that treatment with chlorohydrin and acetylene accelerated germination of lateral buds.

The aim of the work described in this paper is to study the ability of the set to support the growth of the shoot in the absence of any other source of nutrients and with no photosynthesis occurring, and to examine some aspects of the nutritional and physiological interrelationships of the growing regions to the set.

## II. Methods and Materials
### (a) Growth of Sets

One-bud sets were used throughout this work. Sets were germinated in a variety of ways, e.g. by being placed in either a vertical or horizontal position in an atmosphere saturated with water vapour at 30°C (close to the optimal temperature), or being placed on wet bags or in sand culture. For the experiment which provided the analytical results, growth took place in acid-washed quartz sand. The sets were selected from the middle portion of stalks of variety Pindar (clonal material), and were cut at a distance of 5–6 cm on each side of the leaf scar at the node. The sets were numbered, weighed, and dipped in a solution of 0·5 per cent. (w/v) "Aretan"* to minimize fungal attack at the cut ends. The sets were then divided into one group of 60 and four groups of 30 by use of a table of random numbers. The group of 60 was used for the time zero analyses. The remaining

* A commercial organomercurial fungicide.

groups of sets were planted at a depth of 1–1·5 cm and were grown in the dark at a temperature of 30°C, and in an atmosphere saturated with water vapour. Watering was carried out at 2-day intervals throughout the 4-week period of growth.

This experiment was designed along the lines described· by Went (1955) to obviate the necessity of a statistical treatment. The method is to reduce the phenotypic and genotypic variability to a minimum in order to simplify the experiment. These requirements were complied with by careful selection of the starting material and controlling the conditions of growth. The choice of relatively large sample sizes was due to the need to obtain sufficient material for analytical purposes.
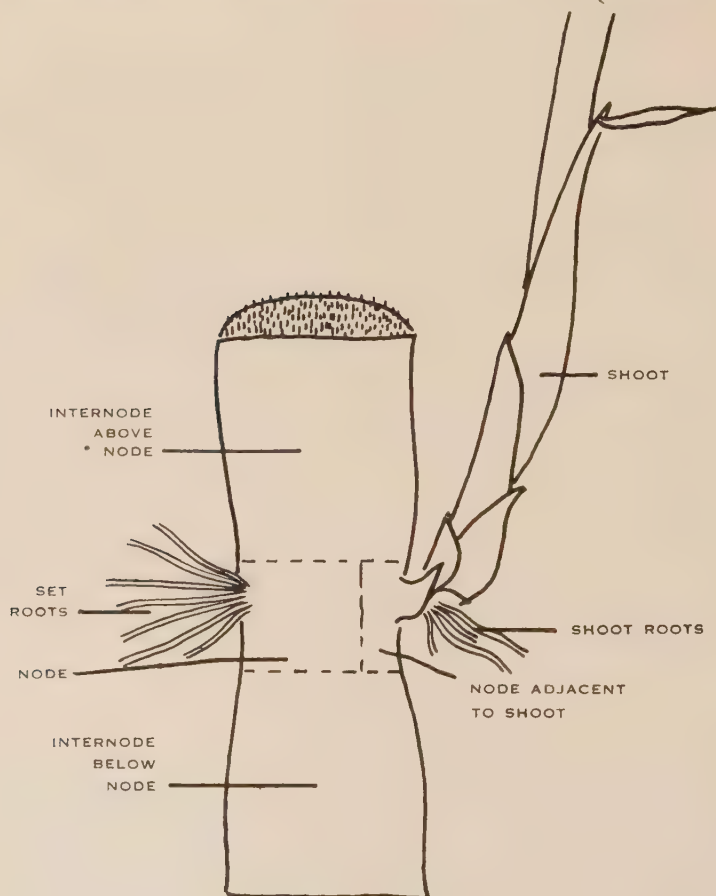
Fig. 1.—Sugar-cane set showing division into regions for analysis.

### (b) Sampling of Material

The sets were divided into regions as shown in Figure 1. The node and internodes were sampled using a cork borer. Four punches were taken from each region except the node adjacent to the bud, from which two punches were taken. The method of sampling excluded the rind, so that the stem material consisted of parenchyma and vascular tissue only.

### (c) Analytical Methods

All the analytical results are means of at least two determinations. Where the error was greater than 5 per cent. the analysis was repeated and the result given is the mean of four or more determinations.

(i) *Drying of Samples.*—Drying was carried out overnight at 70°C, and then *in vacuo* at 70°C for 4 hr. The dried material was homogenized in a Waring Blendor for about 10 min, and stored in sealed bottles until required. Samples taken for analysis were again dried for 2 hr at 70°C *in vacuo*.

(ii) *Phosphorus.*—Phosphorus was determined by the method described by Allen (1940). To measure inorganic phosphorus, the sample was extracted overnight with distilled water, and the phosphorus content in an aliquot determined.

(iii) *Nitrogen.*—Total nitrogen was determined by the method of McKenzie and Wallace (1954). Protein nitrogen was determined after extraction of the sample with 80 per cent. (v/v) ethanol.

(iv) *Soluble Sugars.*—Soluble sugars were determined, after inversion, on an extract of the sample made with 80 per cent. (v/v) ethanol. Glucose was used as a standard. Reducing sugar was determined by the method of Somogyi (1945). Inversion was carried out with $0.67N$ HCl at 67–70°C for 5 min, followed by cooling with water for 30 min.

(v) *Alcohol-insoluble Solids* (*A.I.S.*).—A.I.S. were determined gravimetrically after extraction of the sample with 80 per cent. (v/v) ethanol.

### III. Results

### (a) Germination of Sets

When one-bud sets were placed at 30°C in an atmosphere saturated with water vapour, germination of the root primordia at the root ring in the node usually occurred, but in only about 30 per cent. of sets did the bud show any sign of growth. Prior soaking of the sets increased the percentage of buds germinating under these conditions. Observations showed that there was no correlation between tendency to germinate and the position on the stalk from which the set originated. Tendency for the bud to germinate when no external moisture was supplied appeared to be a property of the individual bud, and was presumably influenced by the physiological conditions in the plant during and subsequent to differentiation of the bud. When a more efficient external supply of moisture was available, such as when sets were placed on a wet bag, or in sand culture, germination of set roots and buds was much more rapid and approached 100 per cent.

### (b) Growth and Moisture Content

Changes in moisture contents and fresh and dry weight of the germinating sets are shown in Figure 2. Germination of the bud was accompanied by a marked increase of moisture content in the first 7 days after planting after which there was a gradual decline. The dry weight of the shoot increased in an approximately linear

fashion with time over the whole of the growth period, which indicates that the growth rate was not limited by a diminution of the concentration of any nutrient (Fig. 2(d)). Such a diminution could be expected to give a curve falling off with time. One explanation for the linear nature of the curve is that an enzyme-catalysed reaction was rate limiting for growth, the concentration of the substrate being sufficiently high to keep the enzyme saturated with substrate molecules during the 4-week growth period, thus giving a zero-order reaction.
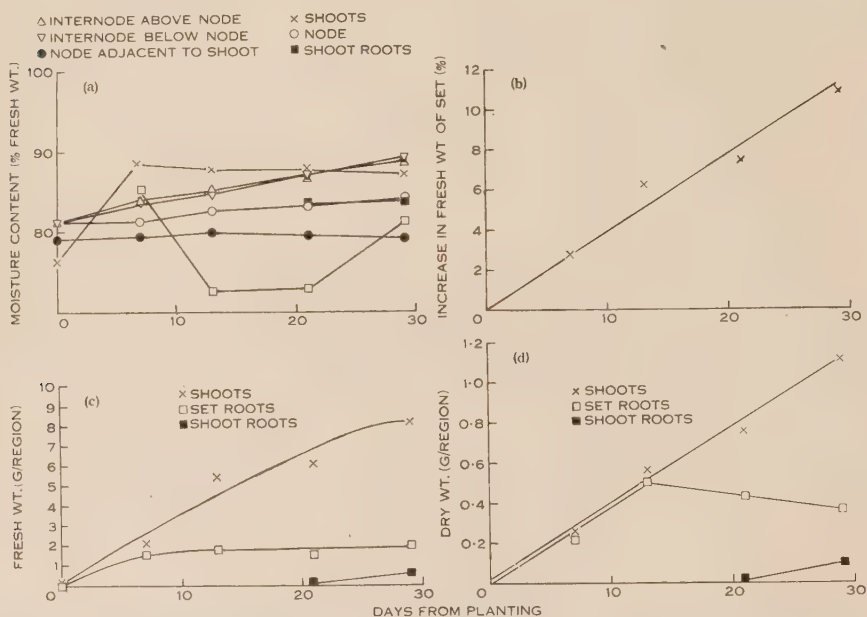


Fig. 2.—Changes in (a) moisture content, (b) fresh wt., (c) fresh wt. per region, and (d) dry wt. per region in germinating sets.

The marked changes occurring in the moisture contents of the set roots (Fig. 2(a)) were correlated initially with secondary thickening (7th–13th day) and then reassimilation, as evidenced by the decrease in total dry weight, and the movement of nitrogen out of this region (Fig. 8(a)). Endogenous respiration would be a correction factor over the whole curve and would account for a portion of the decrease in dry weight of the set roots after the 13th day (Fig. 2(d)). The increases in dry weight of the shoot and the set roots follow an almost identical curve up to the 13th day from planting, after which the curve for the shoot continues in a linear manner while the curve for the set roots shows a marked change from an approximately linear increase in dry weight to a linear decrease (Fig. 2(d)). The first part of the curve for the set roots may reflect a tendency towards a steady state, and the second part their subsequent senescence.

The increase in fresh weight of the set (Fig. 2(b)) could be due only to the uptake of water as no nutrients were available in the growth medium and no photo-synthesis took place. The uptake of water was somewhat greater than that indicated

as the amount of material used in respiration has not been taken into account. Under the conditions of the experiment growth may be considered to be a change in hydration resulting from re-organization of the cellular components. A feature of this re-organization is the continued uptake of water into the internodes and node, despite a considerable loss of solutes from these regions. The movement of solutes could be expected to result in a lowered osmotic pressure of the cell sap. The retention of a diffused pressure deficit in the cells of the internodes and the node over the 4-week period could occur if (1) the set was very slow in equilibrating with the external environment, or (2) if structural changes in the cell walls were occurring which would allow cell expansion. The former explanation is unlikely as the rapid movement of water through the cut ends of the sets can be readily demonstrated by immersing one end in a solution containing a dyestuff. The second

TABLE 1

CHANGES IN ALCOHOL-INSOLUBLE SOLIDS IN REGIONS OF
THE GERMINATING SET

| Region | Alcohol-insoluble Solids (g/region) | |
|---|---|---|
| | Zero Time | 29 Days |
| Internodes | 1·7 | 2·7 |
| Node | 0·84 | 1·5 |
| Node adjacent to shoot | 0·14 | 0·24 |

alternative requires that an increase in cell wall plasticity should occur, and this may be mediated by auxin originating in the growing shoot. Evidence that cell expansion may be followed by new cell wall synthesis is given in Table 1, which shows that A.I.S. of the internodes and node (consisting mainly of cell wall material) actually increased over the growth period. Wilson and Skoog (1954) found that cell expansion in tobacco pith sections cultured on IAA medium was accompanied by a corresponding increase in cell wall material.

### (c) Carbohydrates

The changes in sugar concentrations and A.I.S. content of the various regions of the germinating set are shown in Figures 3 and 4. The decrease in soluble sugars of the internodes and node represents about 9 g glucose over the 4-week period. The total dry weight of the growing regions after 4 weeks was 1·6 g and the increase in dry weight of A.I.S. in the internodes and node was 1·8 g, so that approx. 5·6 g sugar was used up in synthesis of 3·4 g new plant material. Part of this sugar would be utilized for maintenance processes, so the efficiency of the overall changes was quite high.

Following a regular increase in the percentage of A.I.S. in the shoot (Fig. 4(b)), there was a decrease from the 3rd to the 4th week. At that time, the concentration of soluble sugars in the juice in the region node adjacent to the bud levelled off. This region is the doorway to the shoot, and as the concentration of soluble sugars in the juice of the internodes approached the same value as the levelling off value of the region node adjacent to bud it appears that by the 4th week the transfer
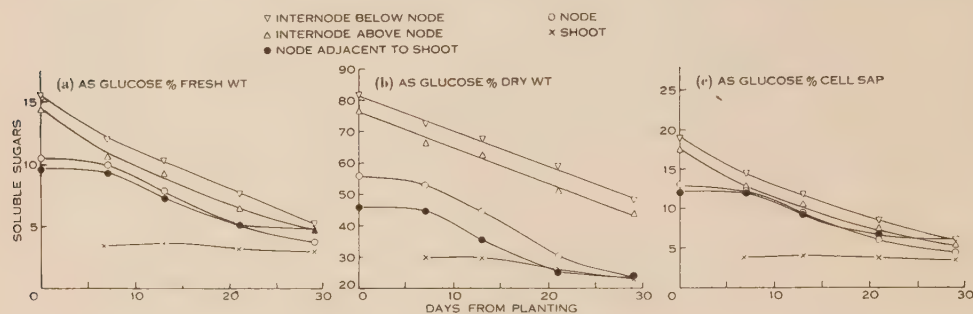


Fig. 3.—Changes in sugar content of regions of germinating sets. Results are for sugars soluble in 80 per cent. ethanol and determined after inversion.

of sugar to the shoot was nearing cessation. Extrapolation in Figure 3(c) (concn. of soluble sugars in juice) indicates that the concentration of sugar in the inter-nodes and the shoot would have been the same at about 40 days, if transfer had continued in the same way. It appears to be likely that the drop in A.I.S. in the shoot represents utilization of polysaccharides under conditions in which the supply of soluble sugar was failing. Part of this polysaccharide may have been starch stored at the growing point as indicated by Went (1896). Studies by Karlsson and Eliasson
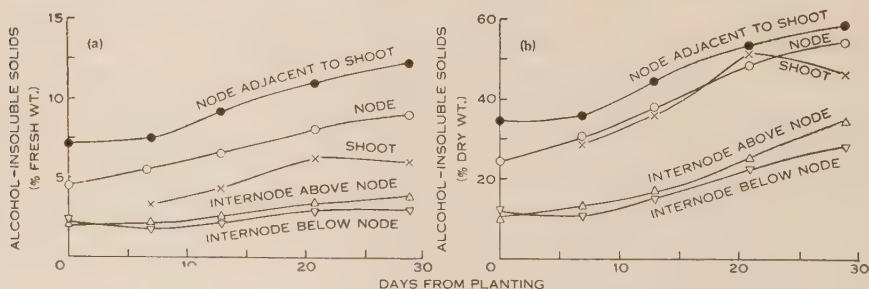


Fig. 4.—Changes in alcohol-insoluble solids in regions of germinating sets.

(1955) on the respiratory quotient of elongating wheat root segments have suggested that, in the absence of other substrate, cell wall dissolution may occur in the extension region with utilization of the products as respiratory substrates.

The concentration of soluble sugars in the juice of the node was less at the end of the 4th week than in the region node adjacent to shoot, but higher than in the shoot itself so that any transfer of sugar from node to shoot would have been across an adverse diffusion gradient. However, it is likely that relatively few of the cells

are functional in this transfer and the movement of sugar against a concentration gradient may be apparent rather than real. These relationships are emphasized when the concentrations of sugars are expressed in terms of a percentage of the juice, less so when expressed as percentage fresh weight, and are obscure when expressed in terms of percentage dry weight.

### (d) *Phosphorus*

The changes in phosphorus levels are shown in Figure 5. Calculation shows that there was approx. $6 \cdot 2$ mg phosphorus in the node and internodes at the time of planting, and $3 \cdot 7$ mg at the end of the 4-week period. The difference would correspond approximately with the phosphorus of the growing regions, the discrepancy being due to the method of sampling which did not include the rind and cells close to it. The node and internodes contributed almost equal quantities of phosphorus to the growing regions, whereas for nitrogen (Section III(*e*)) the internodes were the main source of supply.
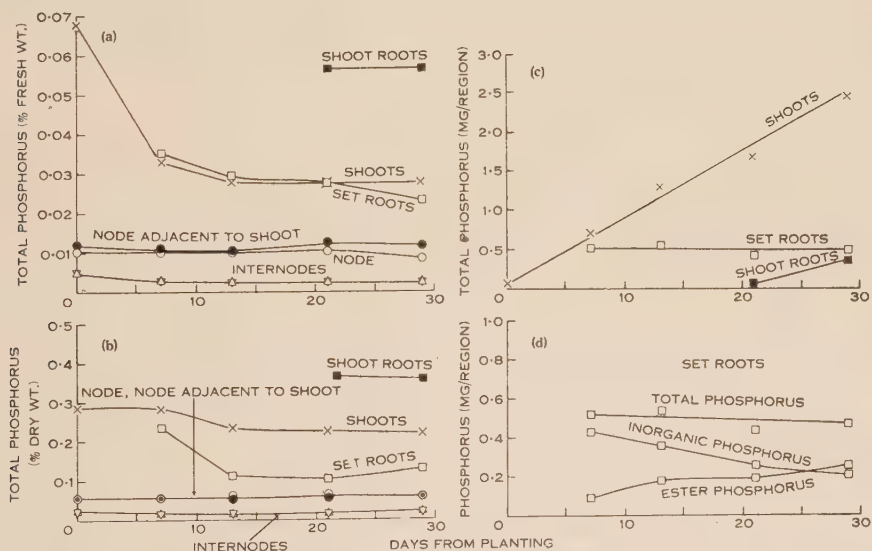


Fig. 5.—Changes in the phosphorus content in regions of germinating sets.

Despite a shifting base line due mainly to the transport of soluble sugars from the internodes, the total phosphorus and total nitrogen remained approximately constant in terms of dry weight, indicating proportionality between the three nutrients in mobilization and movement to the growing regions.

The phosphorus metabolism of the set roots is remarkable in that the total amount of phosphorus (mg/region) remained almost constant over a period in which the dry weight and total nitrogen were increasing sharply (up to the 13th day) and subsequently decreasing. In Figure 5(*d*) the contributions made by inorganic phosphorus and ester phosphorus in the set roots are shown. The ratio of the two components varied considerably over the growth period, and the fact that the sum

of the two remained almost constant may have been fortuitous. It is to be empha-
sized that the figures quoted for ester phosphate probably represent a minimal
value, as no special steps were taken to prevent enzyme action occurring during
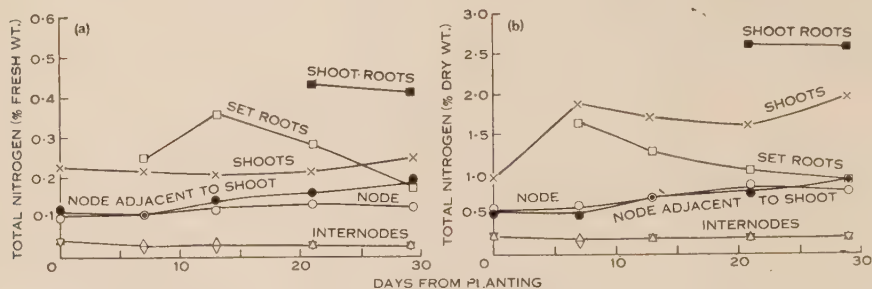


Fig. 6.—Changes in total nitrogen content of regions of germinating sets.

sampling and drying. Also ester phosphate increased throughout the 4-week period
in the set roots in contrast with the disappearance of alcohol-soluble and alcohol-
insoluble nitrogen and also a decrease in dry weight.
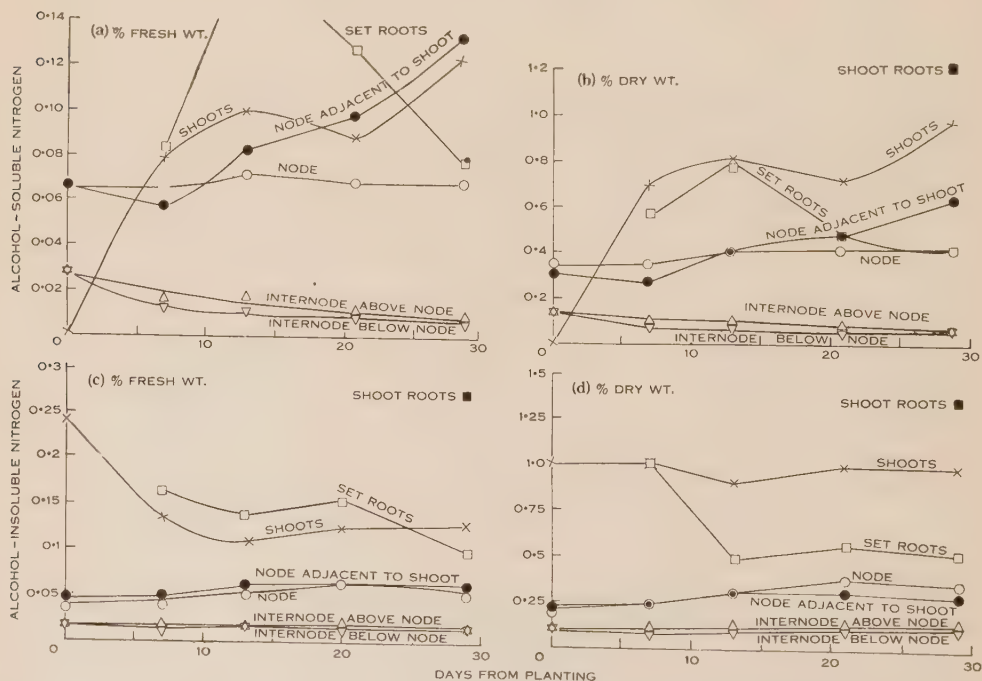


Fig. 7.—Changes in alcohol-soluble nitrogen and alcohol-insoluble nitrogen of regions of
germinating sets.

(e) *Nitrogen*

The results for nitrogen determinations are plotted in Figures 6, 7, and 8.
Approximate calculations show that there would have been 56 mg total nitrogen
in the node and internodes at the time of planting and 43 mg at the end of the

4-week period. In the same time the nitrogen content of the growing regions increased by 26 mg, the discrepancy being apparently due to the method of sampling which did not include the rind and cells close to it. However, the results show that the

TABLE 2

CHANGES IN NITROG N CONTENTS OF THE NODE AND INTERNODES OF SUGAR-CANE SETS

| Region | Nitrogen (mg/region) | | | | | |
| | Zero Time | | | 29 Days | | |
| | Total Nitrogen | Alcohol-insoluble Nitrogen | Alcohol-soluble Nitrogen | Total Nitrogen | Alcohol-insoluble Nitrogen | Alcohol-soluble Nitrogen |
|---|---|---|---|---|---|---|
| Internodes | 35 | 13 | 22 | 18 | 12 | 6 |
| Node adjacent to shoot | 2·6 | 1·1 | 1·5 | 3·6 | 1·1 | 2·5 |
| Node | 19 | 7 | 12 | 22 | 10 | 12 |

internodes supply a considerable amount of nitrogen to the growing regions, and that the source of this nitrogen is the alcohol-soluble fraction, the alcohol-insoluble fraction remaining constant (Table 2).

The total nitrogen of the shoot in terms of mg/region (Fig. 8(b)) increased in an almost linear manner with time, the departures from linearity being due to the alcohol-soluble fraction, and being correlated with the appearance of shoot roots at the end of the 3rd week. The variations in total nitrogen content of the set roots (mg/region) were also due to the alcohol-soluble fraction, the alcohol-insoluble fraction remaining constant until the 3rd week and then decreasing slightly (Fig.
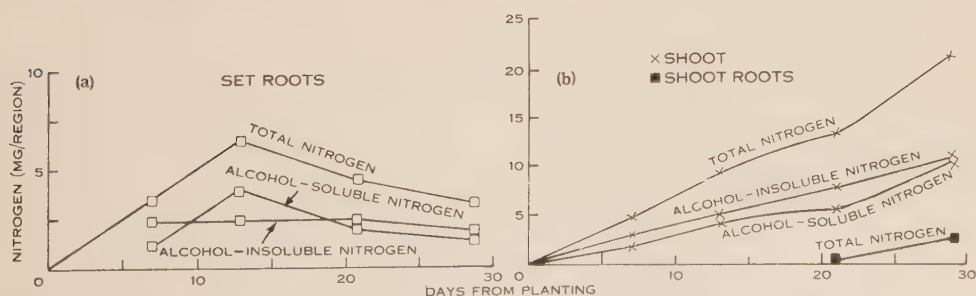


Fig. 8.—Changes in levels of nitrogen fractions in (a) set roots, and (b) shoots from germinating sets.

8(a)). The changes in total and alcohol-soluble nitrogen closely parallel the changes in dry weight of the set roots (Fig. 2(d)) with a linear increase in these components for the first two weeks and then an abrupt change to a linear decrease.

When the results for nitrogen contents are expressed on a fresh or dry weight basis the relationships referred to above are not at all clear, which emphasizes the difficulty of interpreting results when there is shifting basis of reference.

Our finding that virtually all of the nitrogen of the buds at zero time was in the alcohol-insoluble form was further examined by making analyses on different samples of buds from the same variety (Pindar). It was found that the ratio of the two fractions varied somewhat and also that the total nitrogen in terms of percentage of the dry weight was subject to marked fluctuations. Even larger fluctuations (up to 500 per cent.) have been observed in the nitrogen content of the meristematic zone of germinated set roots (Glasziou, unpublished data).

## IV. DISCUSSION

It has become apparent from observations recorded here and elsewhere (see van Dillewijn 1952) that an essential requirement for germination of sugar-cane sets is an uptake of water into the bud and the root primordia of the root ring at the node. Dormancy of the bud and root primordia is evidently due to a delicately poised physiological balance in which auxin plays a major role. In this discussion IAA will be considered to exert its main effect by controlling the plastic and elastic extension properties of the cell walls (Bennet-Clark 1955). It is not implied that this is the only activity of IAA in regulating plant growth, but that such a hypothesis is adequate to explain the results of many experiments on the germination of sugar-cane sets.

Top dominance is normally displayed by sugar-cane; for instance, when cuttings having many buds are planted, only the top buds germinate and these prevent the lower buds, except the lowest, from germinating (Brandes and van Overbeek 1948). However, when single bud cuttings of variety Pindar are germinated, it is found that all buds grow, provided an external supply of water is available. If the sets are placed in an atmosphere saturated with water vapour, germination is sporadic and bears no relationship to the position of the bud on the original stalk. It seems probable that the differences between nodes have been conditioned by environmental factors during differentiation and subsequent maturation.

In a bud which germinates without an external water supply the following things might be expected to occur. Firstly, the destruction of some of the native auxin will free the bud of the inhibitory effect of excess auxin, which may result in an increased plasticity of the cell walls in the bud. The water relationship of the bud to the set may then allow a movement of water from the set and cause cell expansion in the bud of a sufficient degree to trigger cell division in meristematic regions. Philpot and Stanier (1956) have demonstrated that marked changes occur in the nucleus with changing ionic strength of the bathing solution. If the physiological conditions in the bud do not allow a movement of water from set to bud despite increased wall plasticity, germination would occur only if an external water supply was available.

The soaking of sets has long been a practice in sugar-cane culture to promote germination, and much literature on this subject has been reviewed by van Dillewijn

(1952).   Soaking may provide an external water supply as described above, and also leach out some of the excess auxin.   Johnson and Bonner (1956) have shown that portion of the 2, 4-dichlorophenoxyacetic acid taken up by *Avena* coleoptiles from solution is readily leached out when the solution is changed.   It appears to be unnecessary to postulate any more complicated theory than the above to explain these observations made on the germination of buds of sugar-cane sets.

Root primordia in the node may be considered to have a similar delicate poise except that in cuttings the auxin concentration for root development is below the optimum.   If cuttings are placed in a vertical position at 100 per cent. humidity, root development usually occurs but is very slow.   It appears then that the root primordia, although often deficient in auxin, still have their cell walls in an extensible state.   When placed in a saturated atmosphere, transpirational losses may be reduced sufficiently to allow enough water, moving from the set, to extend the cells in the primordium, and thus trigger germination.

TABLE 3

MOVEMENT OF SUGAR FROM THE INTERNODES OF GERMINATING SUGAR-CANE SETS

Columns 1–5 give calculations for fitting data on sugar contents of the internode and shoot to Fick's diffusion equation.   Columns 6–9 give calculations for fitting data for the decrease in sugar concentration in the internodes to an equation for a first-order chemical reaction

| Time (days) | $dM$ | $dM/dt$ | $(C_1-C_2)$ | $\dfrac{dM/dt}{(C_1-C_2)}$ | Time (days) | $C_0/C_t$ | Log $C_0/C_t$ | $\dfrac{\text{Log } C_0/C_t}{T}$ |
|---|---|---|---|---|---|---|---|---|
| 7 | 1·38 | 0·197 | 10·0 | 0·020 | 7 | 18·3/13·5 | 0·132 | 0·019 |
| 6 | 0·65 | 0·108 | 7·0 | 0·015 | 13 | 18·3/11·4 | 0·206 | 0·016 |
| 8 | 1·02 | 0·128 | 4·3 | 0·030 | 21 | 18·3/8·1 | 0·355 | 0·017 |
| 8 | 0·88 | 0·110 | 2·5 | 0·044 | 29 | 18·3/5·5 | 0·523 | 0·018 |

The initiation of germination is accompanied by a movement of nutrients from the cutting to the growing regions, and, for the type of experiment reported here, in which the growing shoot is forced to rely on the cutting for the supply of all of its nutritional requirements, it is remarkable that growth as measured by the increase in dry weight should have continued at a linear rate over a 4-week period. It is therefore unlikely that a depletion in the supply of any nutrient or the diffusion of a nutrient from the set to the shoot was rate limiting for growth.

Went (1896) as a result of studies on the germination of sugar-cane buds suggested that the developing bud exerts an influence on the cutting which results in a conversion of sucrose into glucose, especially in the neighbourhood of the bundles.   The influence exerted by the bud may merely represent a movement of material along a concentration gradient or the bud may actually secrete substances which bring about mobilization of the nutrients available in the set.

In Table 3, the results for the changes in the concentration of sugar in the juice of the internodes has been fitted to Fick's equation:

$$\mathrm{d}M/\mathrm{d}t = PA(C_1 - C_2)/L,$$

where $M$ is the amount of substance moving, $P$ is the permeability constant of the tissue, $A$ is the cross-sectional area, and $(C_1 - C_2)/L$ is the concentration gradient between internode and shoot. If the movement of sugar occurred by a simple diffusion process the term $\mathrm{d}M/\mathrm{d}t \times 1/(C_1 - C_2)$ should be a constant. It can be seen in column 5 of Table 3 that such a treatment was unsatisfactory. However, when the data are fitted to the equation for a first-order chemical reaction

$$\log\ C_0/C_t = KT,$$

where $C_0$ and $C_t$ give the concentrations of sugar of the internode at zero time and time $T$, it can be seen (column 9, Table 3) that the term $(\log C_0/C_t)/T$ is approximately constant. The controlling enzyme could well be invertase, which catalyses a first-order reaction, but there are other possibilities and a thorough analysis of the changes in concentrations of the individual sugars and phosphorylated sugars will be necessary to yield conclusive results. Such an investigation may well throw light on the mode of translocation of sugars in the vascular system.

Certain aspects of the work described in this paper have a bearing on the technique of tissue analysis of sugar-cane for determining the nutritional requirements of the growing crop, a technique which has attracted much attention from plant physiologists interested in the growth of sugar-cane over recent years. A comparison of results for the phosphorus and nitrogen content of the shoot in terms of fresh weight (Figs. 5(a) and 6(a)) shows that the level of phosphorus diminishes sharply and then becomes constant while that of nitrogen remains fairly steady. When the results are compared on a dry weight basis (Figs. 5(b) and 6(b)) it is found that the phosphorus is fairly constant while the nitrogen increases sharply and then levels off. Reference to Figure 5(c) and 8(b) shows that the total amount of phosphorus and nitrogen on a per shoot basis both increased in a more or less linear manner over the whole growth period. These results are for a region which was growing at an approximately constant rate (Fig. 2(d)). When similar comparisons are made for the set roots much more complex interrelationships are evident. In this case the results are further complicated by the change over from a constant growth rate to senescence after 2 weeks. Other examples of the effect of a shifting basis of reference have been pointed out during discussion of results in this paper. In certain cases, interpretation has been assisted when results could be expressed on a per region basis, a possibility which is precluded in many forms of tissue analysis. None of the commonly used bases of reference, including protein nitrogen, would have been completely satisfactory for interpreting the results of this work, and this emphasizes the need for a better reference basis. For sugar-cane the best possibility appears to be deoxyribonucleic acid, but its use awaits development of satisfactory analytical procedures.

## V. Acknowledgments

C.S.I.R.O., and the Botany School, University of Sydney. The author wishes to thank the Management of the Company for permission to publish this paper, Miss D. Small for technical assistance, Dr. R. N. Robertson for advice and guidance throughout the course of the work, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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# NUTRIENT INTERACTIONS AND DEFICIENCY DIAGNOSIS IN THE LETTUCE

## IV. PHOSPHORUS CONTENT AND RESPONSE TO PHOSPHORUS

### By A. E. Grant Lipp* and D. W. Goodall†

### Summary

Lettuce plants grown in sand culture, and receiving nitrogen, phosphorus, and potassium at five levels in all combinations, were analysed at different stages of growth for these elements. An attempt was made to relate these analytical data to the growth response following a supplementary application of phosphorus.

The concentration of phosphorus in the plant dry matter was usually increased by increasing levels of phosphorus supply, decreased by additions of the other nutrients. Increases in phosphorus content were greatest when potassium was deficient or when nitrogen was abundant.

The increase in dry weight of the plants, as a response to additional phosphorus supplied at 46 days from sowing, was closely related to the phosphorus content of the plants previously; there was no clear evidence that this relationship depended on the nitrogen or potassium content of the plants. The date of sampling for analysis (between 29 and 44 days from sowing) did not appear to affect the precision with which response to phosphorus could be forecast. There seemed to be no advantage in analysing selected organs. The relationship between phosphorus content and response was approximately linear.

## I. INTRODUCTION

As was explained in the first paper of this series (Goodall, Grant Lipp, and Slater 1955), the primary purpose of this experiment was to investigate the forecasting of major element deficiencies by chemical analysis of plant material at an early stage of growth. To this end, plants of widely varying nutritional status were grown in sand culture. Samples of these were harvested at several stages, dry weights obtained, and the nitrogen, phosphorus, and potassium contents determined. Further supplies of nutrients were then added, and the resulting increases in dry weight measured with a view to correlating these responses with the chemical analysis.

Previous papers have dealt with the results in terms of dry weight (Goodall *et al.* 1955), water content (Goodall, Slater, and Grant Lipp 1957) and nitrogen relations (Slater and Goodall 1957). The present paper covers the uptake of phosphorus and its concentration in the plant tissues, and the relation between the

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responses to additional supplies of this element on the one hand, and the composition of the plants on the other. A subsequent paper will deal similarly with potassium content and responses.

## II. MATERIALS AND METHODS

Details of the design of the experiment, application and composition of nutrients, and harvesting procedure are given in the first paper. Only a brief summary is included here.

The amounts of the nutrients supplied per pot were as follows:

| Nutrient level | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| N as $NaNO_3$ (mg) | 50 | 200 | 500 | 2000 | 5000 |
| P as $Na_2HPO_4$ (mg) | 2 | 5 | 20 | 100 | 500 |
| K as $K_2SO_4$ (mg) | 0 | 100 | 300 | 1000 | 3000 |

These treatments were applied initially in all combinations, together with an adequate supply of other requisite nutrients. At 46 days after sowing, each of the following subtreatments

| | | |
|---|---|---|
| $S_N$ | 200 mg | N per pot |
| $S_P$ | 20 mg | P per pot |
| $S_K$ | 100 mg | K per pot |

was applied to one of the four pots subjected to each of the 125 initial treatments, the remaining 125 pots being left without subtreatment as controls.

Sample plants were removed for analysis at 11, 22, 29, 37, and 44 days from planting, and the remainder were harvested at 98 days. The analyses at 11 days were performed on the whole plants; on later occasions only the aerial portions were analysed. At 11 and 22 days, the treatments sampled consisted of levels 1, 3, and 5 of each nutrient in all combinations—27 in all. At 29, 37, and 44 days, samples were analysed from all phosphorus levels and levels 1, 2, 3, and 5 of the other nutrients, again in all combinations, making 80 treatments in all. At 44 days, the plants were separated into younger leaves, older leaf laminae, and older midribs, wherever this would yield samples large enough for analysis. At 98 days, the weight of plant material available from each treatment was in many cases very small, and consequently full analytical results could be obtained only for a minority of the treatments.

After dry weight determination the plants were ground and re-dried at 100°C prior to analysis. For the determination of total phosphorus, samples of material weighing approximately 20 mg, and containing 15–100 $\mu$g P were digested with concentrated suphuric acid and 100-volume hydrogen peroxide over a low Bunsen flame. Digests were diluted and aliquots taken for colorimetric determination. These determinations were performed using a modification of Fiske and Subbarow's (1925) method in which $\alpha$-aminonaphtholsulphonic acid was used as the reducing agent. Determinations of soluble phosphorus were also performed, but these data are too incomplete to justify presentation here; the methods and results are reported in detail in the original thesis (Grant Lipp 1952).

Owing to the death of certain plants, the later harvests were incomplete to a greater or lesser extent. Even where plants could be harvested, there was not

TABLE 1

TOTAL PHOSPHORUS CONTENT (PER CENT. OF DRY MATTER) AS AFFECTED BY INITIAL TREATMENTS: ANALYSIS OF VARIANCE

| Days from Sowing | Source of Variation | Degrees of Freedom | Mean Square $\times 10^{-5}$ |
|---|---|---|---|
| 11 | N | 2 | 756 |
| | P | 2 | 9270 |
| | K | 2 | 5129 |
| | N × P | 4 | 1552 |
| | N × K | 4 | 2429 |
| | P × K | 4 | 850 |
| | Error | 3 | 1257 |
| 22 | N | 2 | 1608*** |
| | P | 2 | 23506*** |
| | K | 2 | 3544*** |
| | N × P | 4 | 319** |
| | N × K | 4 | 125 |
| | P × K | 4 | 609** |
| | Error | 8 | 44 |
| 29 | N | 3 | 755 |
| | P | 4 | 40381*** |
| | K | 3 | 8539*** |
| | N × P | 12 | 795* |
| | N × K | 9 | 396 |
| | P × K | 12 | 1192*** |
| | Error | 34 | 289 |
| 37 | N | 3 | 834 |
| | P | 4 | 23298*** |
| | K | 3 | 3335*** |
| | N × P | 12 | 755 |
| | N × K | 9 | 503 |
| | P × K | 12 | 806 |
| | Error | 24 | 400 |
| 44 | N | 3 | 1837*** |
| | P | 4 | 27295*** |
| | K | 3 | 4394*** |
| | N × P | 12 | 926** |
| | N × K | 9 | 270 |
| | P × K | 12 | 1205*** |
| | Error | 33 | 259 |

\* $P$: 0·01–0·05.     \*\* $P$: 0·001–0·01.     \*\*\* $P < 0·001$.

always enough material to permit analysis. In all these cases, "missing plot" values had to be fitted before an analysis of variance was performed; the methods used

TABLE 2

PHOSPHORUS CONTENT (PER CENT. OF DRY MATTER): SIGNIFICANT
EFFECTS OF INITIAL TREATMENTS

22 *Days from Sowing*
Interactions between Initial Treatments

|       | $P_1$  | $P_3$  | $P_5$  |
|-------|--------|--------|--------|
| $N_1$ | 0·239  | 0·465  | 0·551  |
| $N_3$ | 0·245  | 0·376  | 0·579  |
| $N_5$ | 0·180  | 0·329  | 0·504  |
|       |        |        |        |
| $K_1$ | 0·249  | 0·447  | 0·670  |
| $K_3$ | 0·212  | 0·390  | 0·496  |
| $K_5$ | 0·202  | 0·333  | 0·467  |

29 *Days from Sowing*
Interactions between Initial Treatments

|       | $P_1$  | $P_2$  | $P_3$  | $P_4$  | $P_5$  |
|-------|--------|--------|--------|--------|--------|
| $N_1$ | 0·146  | 0·206  | 0·337  | 0·458  | 0·431  |
| $N_2$ | 0·129  | 0·140  | 0·281  | 0·371  | 0·530  |
| $N_3$ | 0·149  | 0·157  | 0·227  | 0·458  | 0·502  |
| $N_5$ | 0·118  | 0·137  | 0·194  | 0·370  | 0·526  |
|       |        |        |        |        |        |
| $K_1$ | 0·155  | 0·201  | 0·328  | 0·588  | 0·678  |
| $K_2$ | 0·126  | 0·154  | 0·248  | 0·374  | 0·442  |
| $K_3$ | 0·132  | 0·152  | 0·253  | 0·402  | 0·426  |
| $K_5$ | 0·130  | 0·134  | 0·210  | 0·292  | 0·452  |

37 *Days from Sowing*
Mean Effects at Different Levels of Initial Treatments

| $P_1$ | 0·096 | $K_1$ | 0·281 |
|-------|-------|-------|-------|
| $P_2$ | 0·126 | $K_2$ | 0·218 |
| $P_3$ | 0·200 | $K_3$ | 0·198 |
| $P_4$ | 0·314 |       |       |
| $P_5$ | 0·377 | $K_5$ | 0·192 |

44 *Days from Sowing*
Interactions between Initial Treatments

|       | $P_1$  | $P_2$  | $P_3$  | $P_4$  | $P_5$  |
|-------|--------|--------|--------|--------|--------|
| $N_1$ | 0·102  | 0·105  | 0·276  | 0·394  | 0·366  |
| $N_2$ | 0·066  | 0·107  | 0·184  | 0·266  | 0·413  |
| $N_3$ | 0·094  | 0·114  | 0·140  | 0·299  | 0·440  |
| $N_5$ | 0·099  | 0·078  | 0·144  | 0·175  | 0·381  |
|       |        |        |        |        |        |
| $K_1$ | 0·086  | 0·100  | 0·245  | 0·392  | 0·583  |
| $K_2$ | 0·084  | 0·089  | 0·186  | 0·245  | 0·322  |
| $K_3$ | 0·093  | 0·115  | 0·172  | 0·282  | 0·350  |
| $K_5$ | 0·099  | 0·100  | 0·141  | 0·215  | 0·356  |

for doing this are described in the original thesis (Grant Lipp 1952), where also the results are presented in greater detail. The data for 98 days were too fragmentary to be worth treatment in this way; although we allude to them in the text wherever appropriate, we have on this account not thought it worthwhile to tabulate them.

## III. Results

### (a) Phosphorus Content as Affected by Treatments

#### (i) Total Phosphorus Content of Plants

Table 1 presents analyses of variance of the data for total phosphorus content at each harvest, as per cent. of dry matter. The mean values for effects shown to be significant are presented in Table 2, and in appropriate cases solid diagrams have been prepared (Figs. 1–5). The mean phosphorus content fell from 0·96 per cent. in the seed to 0·67 per cent. by 11 days and 0·22 per cent. by 37 days. It still remained at about this level at 44 days, but the rather scanty data for 98 days suggest that it subsequently increased.
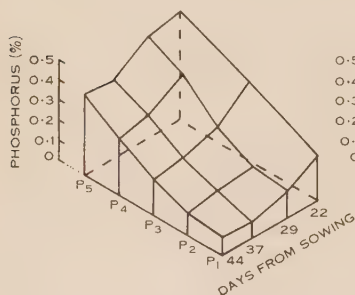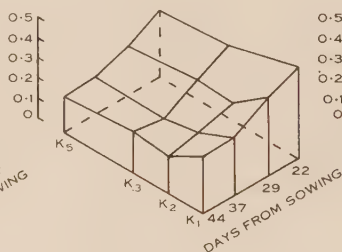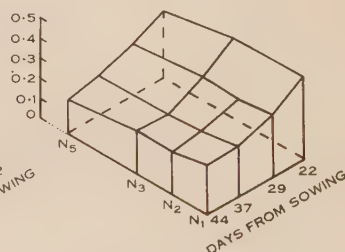


Figs. 1–3.—Effect of phosphorus (Fig. 1), potassium (Fig. 2), and nitrogen (Fig. 3) supply on total phosphorus content at different stages of development.

(1) *Mean effects of initial treatments.*—From 22 days onwards the effect of phosphorus treatment on phosphorus content was highly significant (Fig. 1). A two- to four-fold increase may be seen from $P_1$ to $P_5$. This effect was already apparent at 11 days, though at that early date it had not reached significance; and the same trend continued to 98 days.

The effect of potassium treatment (Fig. 2) was highly significant from 22 days onwards. There was a marked fall in phosphorus content as the supply of potassium was increased from $K_1$ to $K_2$, but further increases in potassium supply had little or no effect. Differences of the same sort could already be seen at 11 days. There was a large increase in dry weight from $K_1$ to $K_2$, and little or no increase as potassium supply was increased further. The fall in phosphorus content from $K_1$ to $K_2$ may thus be regarded as a "dilution" effect.

Nitrogen supply fairly consistently resulted in a decreased phosphorus content (Fig. 3), though this effect reached significance only at 22 and 44 days. This cannot

be explained, like the effect of potassium, in terms of dilution only, since the highest level of nitrogen supply had an adverse effect on yield. This effect of nitrogen on phosphorus content was also apparent in the fragmentary data for the final harvest.

(2) *Interactions of initial treatments.*—The nitrogen–phosphorus interaction (Fig. 4) reached significance at 22, 29, and 44 days. At all times the main feature of this interaction seems to have been a failure of the highest level of phosphorus supply ($P_5$) to increase phosphorus content where nitrogen was deficient. Where the nitrogen supply was adequate, a progressive increase in phosphorus content occurred throughout the range of phosphorus treatments used. At 22 days, this interaction is somewhat obscured by the absence of analytical data for treatments including $P_4$ (the optimum for phosphorus content where nitrogen supply was low); but the data available are quite consistent with this interpretation based on later harvests. At 98 days, too, the results, though in themselves hardly adequate as a basis for firm conclusions, support those derived from fuller data of earlier harvests.
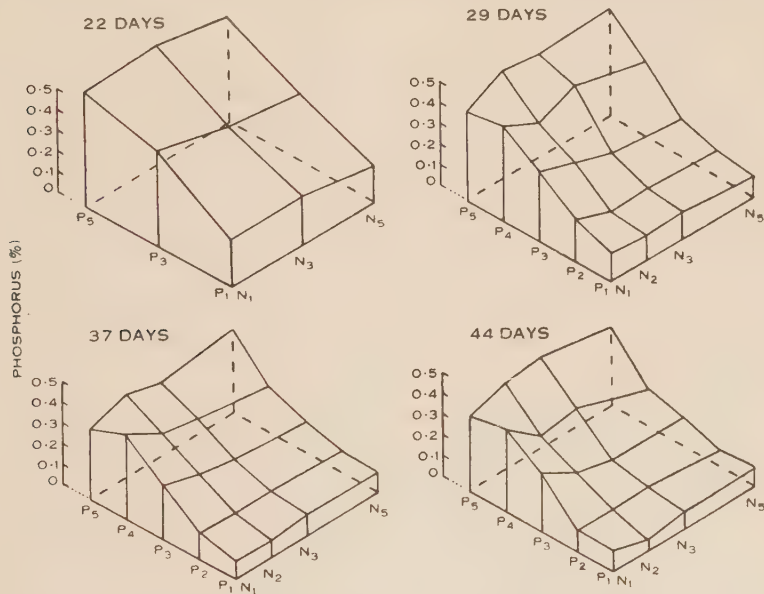


Fig. 4.—Interaction effects of nitrogen and phosphorus supply on total phosphorus content.

The phosphorus–potassium interaction (Fig. 5) was also highly significant at 22, 29, and 44 days. This interaction shows itself in a more marked influence of potassium supply on phosphorus content at the higher levels of phosphorus supply; there is an accumulation of phosphorus with increasing phosphorus supply when growth is restricted by potassium deficiency ($K_1$). Conversely, where phosphorus supply is limiting, potassium deficiency does not result in any appreciable accumulation or "luxury consumption" of phosphorus. At 98 days, the data available are insufficient to show this interaction.

At no stage does the interaction between nitrogen and potassium effects approach significance.

(3) *Subtreatment effects.*—Analytical data at 98 days are too incomplete to allow any firm conclusions to be reached, but at least it may be said that supplementary phosphorus at 46 days increased the content of phosphorus in plants initially receiving a low supply of this element. No general effect of supplementary nitrogen or potassium on phosphorus content was recognizable.

(ii) *Total Phosphorus Content of Separated Organs*

As already indicated, the plants harvested at 44 days were divided into younger and older leaves wherever they were large enough, and the latter were further divided into laminae and midribs. These organs were analysed separately, and the data thus available covered 24 initial treatments—unfortunately excluding all the low-phosphorus treatments, in which the plants were too small for separation. The data for midribs were incomplete even within this limited range of treatments; the data for the other separated organs were subjected to analysis of variance, the results of which are presented in Table 3. Table 4 presents the means for the major differences observed.
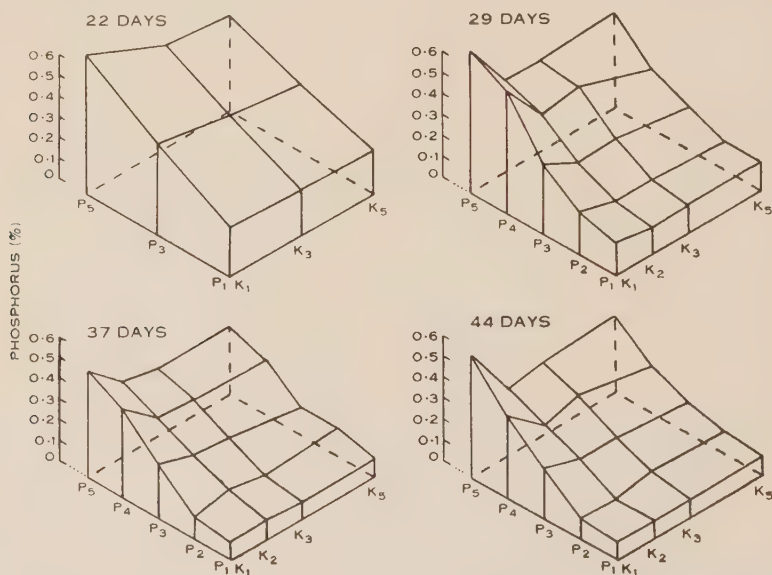


Fig. 5.—Interaction effects of potassium and phosphorus supply on total phosphorus content.

There were fairly consistent differences in the phosphorus content of the different organs, the younger leaves containing nearly twice as much (on a dry weight basis) as the older leaf laminae, while the midribs contained even less than the latter (for those treatments for which full data were available, the means were respectively $0.480$, $0.286$, and $0.203$ per cent.). In the effects of treatments, however, the organs did not differ greatly from one another, or from the aerial parts as a whole. The increase in phosphorus supply from $P_3$ to $P_5$ led to a very substantial increase in the phosphorus content of all parts, while supply of potassium decreased the phosphorus content—perhaps rather more markedly in the older than the younger leaves.

TABLE 3

TOTAL PHOSPHORUS CONTENT (PER CENT. OF DRY MATTER) OF
SEPARATED ORGANS AT 44 DAYS: ANALYSIS OF VARIANCE

|  | Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|---|
| Younger leaves | N | 3 | 0·0040 |
|  | P | 1 | 0·7643*** |
|  | K | 2 | 0·1326** |
|  | N × P | 3 | 0·0330 |
|  | N × K | 6 | 0·0075 |
|  | P × K | 2 | 0·0132 |
|  | Error | 6 | 0·0113 |
| Older leaf laminae | N | 3 | 0·0026 |
|  | P | 1 | 0·3673*** |
|  | K | 2 | 0·0690** |
|  | N × P | 3 | 0·0197 |
|  | N × K | 6 | 0·0038 |
|  | P × K | 2 | 0·0279* |
|  | Error | 6 | 0·0048 |

*$P$: 0·01–0·05.    **$P$: 0·001–0·01.    ***$P < 0·001$.

TABLE 4

TOTAL PHOSPHORUS CONTENT (PER CENT. OF DRY MATTER) OF
SEPARATED ORGANS AT 44 DAYS

*Younger Leaves*

Mean Effects at Different Levels of Initial Treatments

| $N_1$ | 0·468 |  |  | $K_1$ | 0·610 |
|---|---|---|---|---|---|
| $N_2$ | 0·493 |  |  |  |  |
| $N_3$ | 0·431 | $P_3$ | 0·284 | $K_3$ | 0·402 |
| $N_5$ | 0·456 | $P_5$ | 0·640 | $K_5$ | 0·374 |

*Older Leaf Laminae*

Mean Effects at Different Levels of Initial Treatments

| $N_1$ | 0·252 |  |  | $K_1$ | 0·376 |
|---|---|---|---|---|---|
| $N_2$ | 0·286 |  |  |  |  |
| $N_3$ | 0·289 | $P_3$ | 0·146 | $K_3$ | 0·224 |
| $N_5$ | 0·251 | $P_5$ | 0·393 | $K_5$ | 0·208 |

Interactions between Initial Treatments

|  | $K_1$ | $K_3$ | $K_5$ |
|---|---|---|---|
| $P_3$ | 0·184 | 0·136 | 0·117 |
| $P_5$ | 0·568 | 0·312 | 0·300 |

## (b) Phosphorus Uptake

The amount of phosphorus in the tops (or, at 11 days, in the whole plants) was calculated from the data for dry weight and per cent. phosphorus in plants receiving the 1, 3, and 5 levels of each nutrient. These figures provide an indication of the rate of uptake from the substrate, the error introduced by failure to include the roots in later harvests being small.

The course of phosphorus uptake with different levels of phosphorus supply is shown in Figure 6. During the first 11 days, the plants were growing at the expense of phosphorus stored in the seed, for in no treatment was the phosphorus contained in the plants at the first harvest appreciably more than that in the seed.
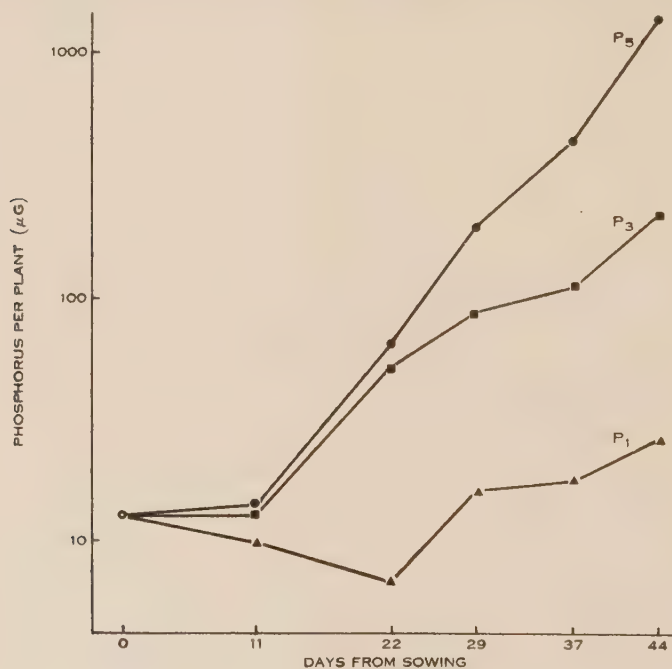


Fig. 6.—Course of uptake of phosphorus.

By 22 days, those receiving the larger amounts of phosphorus had begun to make use of them, but the plants from the $P_1$ treatments were still existing entirely on seed phosphorus. The decrease here may be accounted for by the phosphorus in the roots, which were included at 11 days but not at later stages. Uptake by plants in the $P_1$ treatments continued to be slow, so that even after 98 days the amount in the plants did not exceed four times the seed content, unless supplementary phosphorus had been supplied at 46 days. Even at this extreme level of deficiency, over half of the phosphorus supplied remained unabsorbed; presumably the root system was so small that the medium could not be effectively explored.

Examination of the data for phosphorus uptake and dry weight accumulation emphasizes the phosphorus starvation of the plants under the $P_1$ treatments. Thus, with $P_1$, dry weight per plant increased by a factor of 10 and phosphorus content

per plant only by a factor of five between 22 and 44 days, and consequently the phosphorus concentration in the plant tissue decreased to half its value over the same period. On the other hand, with $P_5$, phosphorus uptake and growth kept nearly in step; both increased about 30-fold, and the phosphorus content declined only slightly over this period.

Generally, increased supplies of nitrogen and potassium resulted in increases in phosphorus uptake, especially at the higher levels of phosphorus supply, by allowing greater growth to take place. Uptake was not reduced to the same extent as dry weight by supra-optimal levels of these nutrients.

TABLE 5
GROWTH RESPONSE TO PHOSPHORUS (MG)

| Nitrogen Levels | Potassium Levels | Phosphorus Levels | | | | |
|---|---|---|---|---|---|---|
| | | $P_1$ | $P_2$ | $P_3$ | $P_4$ | $P_5$ |
| $N_1$ | $K_1$ | +895 | −175 | −136 | −134 | −222 |
| | $K_2$ | +730 | +993 | +961 | — | −794 |
| | $K_3$ | +1033 | +39 | +33 | −190 | −342 |
| | $K_5$ | +740 | +518 | −378 | −205 | −101 |
| $N_2$ | $K_1$ | +77 | +152 | +444 | — | −559 |
| | $K_2$ | +1580 | +665 | +1786 | −1166 | −692 |
| | $K_3$ | +1463 | +2141 | +4246 | +532 | −1294 |
| | $K_5$ | +1492 | +3972 | +2065 | −1170 | −63 |
| $N_3$ | $K_1$ | +225 | +3737 | +676 | +170 | −71 |
| | $K_2$ | +1991 | +1786 | +967 | — | +237 |
| | $K_3$ | +1147 | +2280 | +220 | −530 | −3192 |
| | $K_5$ | +7486 | +1780 | +3366 | −1355 | +1616 |
| $N_5$ | $K_1$ | +281 | +370 | +82 | −9 | −136 |
| | $K_2$ | −88 | +111 | +451 | +645 | +322 |
| | $K_3$ | +2 | +829 | +2446 | −2033 | −1431 |
| | $K_5$ | +519 | — | — | +4605 | +2567 |

As already mentioned, the total amount of phosphorus removed from the medium by plants receiving the $P_1$ treatments did not exceed 1 mg per pot—i.e. half the amount supplied. At higher phosphorus levels, the proportion of the phosphorus supplied that was taken up by the plants was less, and rarely reached 20 per cent.

(c) *Relationship between Growth Response to Phosphorus and Chemical Composition*

Responses to the phosphorus subtreatment were estimated, for each initial treatment, by the difference in dry weight between the plant receiving this subtreatment and its control (Table 5). These differences were corrected for the differences in dimensions of the two plants at the time of applying the subtreatment, as described previously (Goodall *et al.* 1955).

Regression methods were then used to study the extent to which analytical data could be used to predict the responses to the phosphorus subtreatment. Besides the figures for phosphorus content reported in this paper, data on nitrogen (Slater and Goodall 1957) and potassium (Grant Lipp 1952) were also available. Since material from treatments including $N_4$ and $K_4$ had not been analysed, only 80 initial treatments came into consideration; and of these a few could not be used in the regression study because data for dry weight were missing, either for the subtreatment or the control, or because the analytical data included fitted values.

TABLE 6

ANALYSIS OF VARIANCE OF THE REGRESSION OF RESPONSE ON THE COMPOSITION OF ENTIRE AERIAL PARTS

The mean square for reduction due to regression was tested in each case against the residual mean square for that regression. The effect of reducing predicting variables from three to two (or to one), given by the difference in the sum of squares due to regression divided by one (or two) degrees of freedom, can be tested against the residual mean square fitting N, P, and K

| Reduction due to Regression on: | Degrees of Freedom | 29 Days | | 37 Days | | 44 Days | |
|---|---|---|---|---|---|---|---|
| | | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ |
| N, P, and K | 3 | 3390 | 1130** | 3675 | 1225** | 4365 | 1455*** |
| N and P | 2 | 3372 | 1686** | 3430 | 1715** | 3639 | 1820*** |
| N and K | 2 | 2747 | 1373** | 1270 | 635 | 1933 | 966* |
| P and K | 2 | 3278 | 1639** | 3470 | 1735** | 3682 | 1841*** |
| N alone | 1 | 2743 | 2743** | 1005 | 1005* | 422 | 422 |
| P alone | 1 | 3266 | 3266*** | 3386 | 3386*** | 3211 | 3211*** |
| K alone | 1 | 120 | 120 | 715 | 715 | 1929 | 1929** |
| Residue fitting N, P, and K | 63 | 14994 | 238 | 14709 | 234 | 14019 | 223 |

*P: 0·01–0·05.　　**P: 0·001–0·01.　　***P < 0·001.

(i) *Total Phosphorus.*—At 29, 37, and 44 days, where a substantial number (67) of complete sets of data was available, the regression of response on the total phosphorus content of the aerial parts was highly significant (see Table 6), and the closeness of this relationship did not change with time. The significant regressions on nitrogen content may be ascribed to the close relationship between nitrogen and phosphorus content in the earlier harvests. At 44 days the simple regression on potassium content was also significant. The contribution to the multiple regression from nitrogen and potassium content did not reach significance at any stage; this contribution increased, however, as the time of sampling approached that at which the subtreatment was applied, and at 44 days it was probably too large to ignore safely. This contribution falling just short of significance cannot, however, be ascribed either to nitrogen or to potassium separately.

The equations for the simple regressions on phosphorus content are:
for 29 days

$$y = 1987 - 4220\, x_P,$$

for 37 days

$$y = 1954 - 5350\, x_P,$$

and for 44 days

$$y = 1812 - 4967\, x_P,$$

while the multiple regression on the content of all three nutrients at 44 days is:

$$y = -415 + 636\, x_N - 5786\, x_P + 224\, x_K,$$

where $y$ is the response in mg, while $x_N$, $x_P$, and $x_K$ represent the content of the three elements in the plant material (per cent. of dry matter).

TABLE 7

ANALYSIS OF VARIANCE OF THE REGRESSION OF RESPONSE ON THE COMPOSITION OF DIFFERENT
PLANT PARTS AT 44 DAYS

| Reduction due to Regression on: | Degrees of Freedom | Laminae of Older Leaves | | Midribs of Older Leaves | | Younger Leaves | | Entire Aerial Parts | |
|---|---|---|---|---|---|---|---|---|---|
| | | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ |
| N, P, and K | 3 | 2134 | 711 | 1656 | 552 | 1731 | 577 | 2058 | 686 |
| N alone | 1 | 82 | 82 | 5 | 5 | 325 | 325 | 70 | 70 |
| P alone | 1 | 1256 | 1256* | 982 | 982 | 1480 | 1480* | 1299 | 1299* |
| K alone | 1 | 1452 | 1452* | 675 | 675 | 436 | 436 | 1006 | 1006 |
| Residue fitting N, P, and K | 28 | 7322 | 262 | 7801 | 279 | 7725 | 276 | 7398 | 264 |

*$P$: $0\cdot01$–$0\cdot05$.

At 11 and 22 days the number of complete sets of data available was much smaller (19), and the regressions did not reach significance. This does not, however, imply that prediction of response from analysis at this early stage is necessarily inferior to that from analyses at 29 days or later; when corresponding small sets of data were used for these later dates of sampling, the regressions again failed to reach significance.

(ii) *Choice of Organ.*—Regressions similar to those on the analytical data for the entire aerial parts were also computed using analyses of the separated organs at 44 days (Table 7). Unfortunately, only 32 complete sets of data could be used and these did not include treatments with $P_1$ and $P_2$ where responses were most marked. Nevertheless, the regression on phosphorus content was significant both for the laminae of the older leaves and for the entire young leaves, and in the latter case was slightly more significant than that on the analyses for the aerial parts as a

whole, when the corresponding set of data was used. The regression equations are:
for laminae of older leaves

$$y = 1456 - 4139\, x_P,$$

for younger leaves

$$y = 1832 - 3212\, x_P,$$

and for entire aerial parts

$$y = 1603 - 4282\, x_P.$$

In the separated organs, there was no evidence that the nitrogen or potassium content affected the relationship between phosphorus content and response.

TABLE 8

CURVILINEAR REGRESSIONS OF PHOSPHORUS RESPONSE ON PHOSPHORUS
CONTENT AT DIFFERENT TIMES

|  | Degrees of Freedom | Mean Square $\times 10^{-3}$ | | |
|---|---|---|---|---|
|  |  | 29 Days | 37 Days | 44 Days |
| Linear regression | 1 | 29401*** | 30147*** | 26669*** |
| Parabolic term | 1 | 6960 | 4854 | 4785 |
| Remainder | 70 | 2147 | 2167 | 2217 |

*** $P < 0.001$.

## IV. Discussion

In view of the very marked effects of phosphorus treatment on growth, it was to be expected that the phosphorus content of the tissue should be increased. In fact, it was approximately quadrupled by the highest level of treatment at the later harvests, as compared with $P_1$. The increase was already quite marked at 22 days; the suggestion in the figures for 11 days (0·56 per cent. phosphorus for $P_1$ treatments, 0·76 per cent. phosphorus for $P_5$ treatments) that a similar difference existed at this early stage must probably be discarded since the total phosphorus in the plant at that time hardly exceeded the seed content.

Addition of the other nutrients usually reduced the phosphorus content of the tissue. In the case of potassium this reduction may clearly be interpreted as an effect of increased growth, for both changes ceased at the same level of potassium supply, and the decrease in phosphorus content did not occur where phosphorus supply was itself the main factor controlling growth. In the case of nitrogen some more direct effect on phosphate uptake would appear also to be involved, since one can point to several instances where the increase in nitrogen supply from the $N_3$ to the $N_5$ level has resulted in a decrease both in dry weight and in phosphorus content.

Whereas an efficient forecast of the response to supplementary nitrogen required data for leaf phosphorus as well as nitrogen (Slater and Goodall 1957), phosphorus content alone formed an adequate basis for forecasting the response to supplementary phosphorus.   In considering this difference, however, it must be remembered that phosphorus effects were preponderant throughout the investigation; one may surmise that, if the nitrogen treatments had covered a wider range, information on the nitrogen content of the leaves might well have added to the precision with which responses to phosphorus could be forecast.   Lundegårdh (1941, 1951) found this to be true in oats—a given phosphorus content indicated smaller responses to phosphorus if associated with low nitrogen figures than with higher ones.
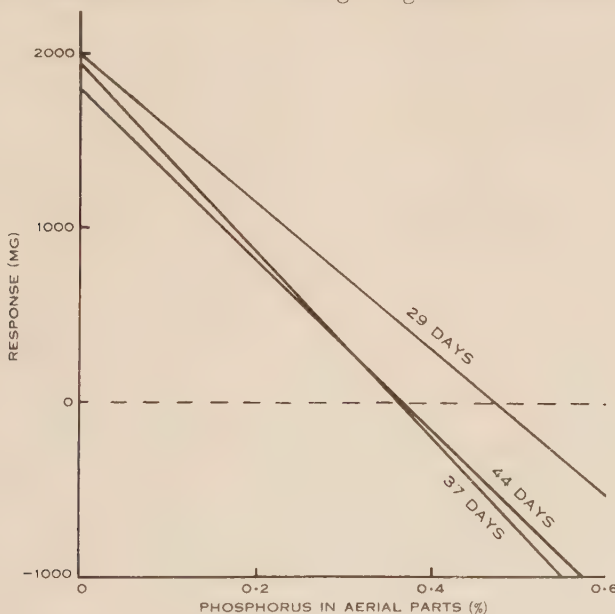


Fig. 7.—Response to phosphorus as a function of phosphorus content.

It was shown (Slater and Goodall 1957) that the relationship between nitrogen response and leaf composition was curvilinear; in oats, Lundegårdh (1941, 1951) found the same to be true for phosphorus responses.   The possibility that the relationship between phosphorus responses and phosphorus content was also curvilinear in the present data was likewise tested.   It was found that, in this case, a quadratic equation did not fit the data significantly better than a linear one.   The results are presented in Table 8, in the form of analyses of variance.

The regression lines for the three dates of sampling are shown in Figure 7; they lead to the following critical values for phosphorus content, above which no positive response to phosphorus supply may be expected: 29 days, 0·471 per cent. phosphorus; 37 days, 0·365 per cent. phosphorus; and 44 days, 0·365 per cent. phosphorus.

Data for the phosphorus content of different organs, and for soluble phosphorus, were rather scanty, but did not suggest that they would have provided a better

forecast of phosphorus response than the total phosphorus content of the tops as a whole. In particular, the data for midribs seemed to form, if anything, an inferior index—in contradistinction to the claims often made (e.g. Emmert 1935) that these organs, containing a large proportion of conducting tissue, should provide a particularly good basis for estimating nutrient status.

## V. Acknowledgments

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# THE CAUSES OF NATURAL DURABILITY IN TIMBER

## I. THE ROLE OF TOXIC EXTRACTIVES IN THE RESISTANCE OF TALLOWWOOD (EUCALYPTUS MICROCORYS F. MUELL.) TO DECAY

By E. W. B. Da Costa* and P. Rudman*

*Summary*

The hypothesis that the natural resistance of certain timbers to decay is due to the deposition in the heartwood of materials toxic to fungi is discussed, and criteria for assessing the role of these materials are outlined. These criteria have been applied in a study of the decay resistance of the highly durable Australian timber tallowwood (*Eucalyptus microcorys* F. Muell.).

Sawdusts from the outer heartwood of four trees of tallowwood were extracted successively with ether, methanol, acetone, water, and dilute alkali. Unextracted and ether-extracted sawdusts were highly resistant to decay by *Trametes lilacino-gilva* Berk., *Coniophora cerebella* Pers., *Coriolus versicolor* (Fr.) Quel., and *Fomes durus* (Jungh.) G. H. Cunn. The methanol extraction greatly reduced decay resistance for all trees and all fungi; the extractions with acetone, water, and dilute alkali had no consistent effect.

The material removed by each solvent was incorporated into heartwood sawdust from the decay-susceptible mountain ash (*E. regnans* F. Muell.) in concentrations (w/w) equal to those in tallowwood. Decay tests showed the methanol extract (12–15 per cent. of oven dry weight) to be highly toxic to all test fungi (as was confirmed by toxicity tests in agar), whereas other extracts showed only slight indications of toxicity.

It is concluded that the high resistance of tallowwood to decay is due almost entirely to the presence of a toxic methanol-soluble material (or materials) and further work to isolate this material is in progress.

## I. Introduction

Although it has been long recognized that some timbers are much less readily attacked than others by such biological agents as wood-rotting fungi, termites, wood-boring insects, and marine organisms, very little is known of the chemical and physical properties of the wood which are responsible for these differences. The high resistance of some timbers to decay is, however, generally ascribed to the deposition in the heartwood of materials toxic to fungi. Though this theory has been held for a long time, little experimental work was done until comparatively recent years, but a number of investigations have now been made and have been partly reviewed by Erdtman (1952, 1955) and by Findlay (1957).

Consideration of the results of these investigations shows that several substances toxic to wood-destroying fungi have been isolated from the heartwoods of durable timber species. In some cases, variations in concentration of the toxic extractives have been shown to be correlated with measured variations in decay

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resistance, but it has not been proved conclusively that the toxic materials isolated give a complete explanation of the decay resistance of the timber. For conclusive proof, it would be desirable to show, firstly, that the extractive concerned will inhibit decay, secondly, that their complete removal from a durable timber renders it highly susceptible to decay, and thirdly, that the decay resistance of individual samples of the timber can be quantitatively related to their content of the specific extractives.

Since our present investigations are concerned with explaining the variations in durability of timber, it was decided to commence with a detailed investigation of the role of various extractives in the decay resistance of one highly durable timber. Tallowwood is one of the most important commercially, and most durable, Australian timbers and is obtained from one clearly defined botanical species, *Eucalyptus microcorys* F. Muell. Laboratory tests had confirmed its reputedly high resistance to decay and had suggested that this resistance was due to toxic extractives. The chemistry of tallowwood has not previously been studied from this aspect, although Cox, King, and King (1956) have isolated from the heartwood a petroleum ether-soluble material which could be saponified to give a steroid which they named *cyclo*eucalenol.

## II. Materials and Methods

### (a) Selection and Preparation of Wood Samples

Material for this investigation was obtained from four large mature trees of tallowwood, each of which came from a different district. A billet, 5 by 5 in. in cross section, was taken from the outer heartwood near the butt of each tree and air dried. A sample representing the entire cross section of the billet was reduced to fine sawdust in a Wiley mill fitted with a 2-mm screen. Decay-susceptible sawdust, into which the tallowwood extracts could be incorporated to study their inhibitory effect on wood-destroying fungi, was similarly prepared from the outer heartwood of mountain ash, *E. regnans* F. Muell., which has a relatively low resistance to decay and is moderately absorbent.

### (b) Test Fungi

The four wood-destroying fungi used for decay tests were:

(i) *Trametes lilacino-gilva* Berk., strain DFP 1109, isolated 1944 from sporophore on *Eucalyptus* sp. An Australasian brown-rot fungus occurring mainly on fallen eucalypt timber; a common cause of destruction of eucalypt timber in service.

(ii) *Coniophora cerebella* Pers., strain DFP 1779, isolated 1938 from decay in *E. marginata* Sm. A cosmopolitan brown-rot fungus, causing both heart rot in standing trees and decay of timber in service; extremely destructive to any kind of timber.

(iii) *Coriolus versicolor* (Fr.) Quel., strain DFP 2666, isolated 1950 from sporophore. A cosmopolitan white-rot fungus, commonly attacking the sapwood of fallen eucalypt logs, but not regarded as capable of severe attack on eucalypt heartwood.

(iv) *Fomes durus* (Jungh.) G. H. Cunn., strain DFP 3882, isolated 1953 from sporophore on *Sterculia laurifolia*. A white-rot fungus, recorded in Australia on rain-forest species in tropical areas; probable effect on eucalypt heartwood not known.

### (c) *Preparation of Extracts*

A 60-g sample of air dried sawdust (of known moisture content) from each tree was taken, weighed into a Soxhlet thimble, and extracted with ether until no more material was being dissolved. The sawdust was then air dried and weighed and one-quarter set aside for decay tests. The remainder was placed in a Soxhlet thimble for extraction with methanol. The ether, methanol-extracted sawdust was air dried and weighed and one-third set aside for decay tests, the remainder being used for acetone extraction. After this stage, a water extraction was carried out on half the remaining sawdust. In this way, approximately equal amounts of ether-extracted sawdust, ether and methanol-extracted sawdust, and so on, were obtained for decay tests.

A known fraction of each extract was set aside for removal of solvent followed by weighing to enable estimation of the concentration (w/w) of each extract in tallowwood on an oven-dry basis. The remainder of the extract was concentrated to a small volume ($2n$ ml) and added to a predetermined weight ($n$ g) of air dried mountain ash sawdust and thoroughly incorporated therein. The impregnated sawdust was then dried slowly at room temperature, being stirred periodically to prevent any concentration of solute at the surface of the sawdust. The weight of mountain ash sawdust impregnated was calculated so that the final concentration of the specific extract (w/w) in the impregnated mountain ash was the same as that in the untreated tallowwood.

The general scheme of successive extractions, impregnations, and selection of samples for decay tests is set out diagrammatically in Table 1.

A final extraction with 0·01N NaOH (200 ml/10 g sawdust) was carried out on a separately prepared sample of ether, methanol, acetone, and water-extracted tallowwood (of known weight and moisture content) in a boiling water-bath. After 2 hr the alkali-extracted sawdust was filtered off in a sintered-glass crucible, washed, and suspended in distilled water. The pH was adjusted to 5·4–5·6 with hydrochloric acid and the sawdust filtered, dried to constant weight, and a moisture content determination made, thus enabling the percentage of alkali-soluble material to be determined by the loss in weight. The pH of the extract was adjusted to 5·0 with hydrochloric acid; the extract was then concentrated under vacuum and impregnated into the predetermined amount of mountain ash. The amount of alkali used was kept to a low level because the presence of any appreciable amount of salt in the neutralized alkali extract would tend to inhibit the growth of the test fungi. In the case of tree MI7, the amount of alkali used may possibly have been insufficient to remove all the alkali-soluble material present.

The extracted tallowwood and impregnated mountain ash sawdusts were air dried and stored at room temperature pending decay tests. Preliminary tests on mountain ash sawdust had failed to show any toxic effect from residual solvent with

TABLE 1

SCHEDULE FOR EXTRACTION AND TESTING OF TALLOWWOOD SAWDUST

Untreated sawdust

Decay tests

ether

Ether-extracted sawdust          Ether extract

1 part

Decay tests

3 parts

methanol     Weight determination     Into mountain ash for decay tests

Ether, methanol-extracted sawdust       Methanol extract

1 part

Decay tests

2 parts

acetone     Weight determination     Into mountain ash for decay tests

Ether, methanol, acetone-extracted sawdust       Acetone extract

1 part

Decay tests

1 part

water     Weight determination     Into mountain ash for decay tests

Ether, methanol, acetone, water-extracted sawdust       Water extract

Decay tests

Weight determination     Into mountain ash for decay tests

this method, but some anomalous results in the main tests suggested such an effect. Part of the test was therefore repeated on sawdusts from the same sources which had been vacuum dried at 40°C for 48 hr; no anomalous results were found in the repeated tests.

The pH of sawdust from each of the four trees of tallowwood was determined by adding distilled water (50 ml/g) and recording the equilibrium figure reached after cessation of stirring. Determinations were carried out in a like manner on ether, methanol-extracted and on ether, methanol, acetone, water-extracted tallowwood, and on the aqueous suspensions of the methanol extracts (1 g/50 ml water).

### (d) Decay Tests

The relative susceptibility to decay of the various types of sawdust was assessed by the percentage loss in oven dry weight obtained when small samples of each sawdust in perforated aluminium dishes were incubated for several weeks in close contact with the test fungi in pure culture. The test fungi were grown on nutrified "feeder strips" resting on moist soil in glass jars, using a technique similar to the "soil block" technique developed by Leutritz (1946) for measuring decay resistance of wood blocks.

The culture jars each contained approximately 100 g of a clay loam soil of exceptionally high organic matter content and water-holding capacity, with a moisture content of 60 per cent., below which capillary movement of water is negligible with this soil. The feeder strips were $1\frac{1}{2}$-in. squares of $\frac{1}{16}$-in. coachwood (*Ceratopetalum apetalum* D. Don) veneer, soaked in a nutrient solution based on that of Jennison, Newcomb, and Henderson (1955), with the composition: casein hydrolysate, 0·08 per cent.; $KH_2PO_4$, 0·15 per cent.; $MgSO_4 . 7H_2O$, 0·05 per cent.; thiamin hydrochloride, 0·001 per cent.; sucrose, 2 per cent. The jars were autoclaved for 2 hr at 15 lb/sq. in., inoculated with the test fungi, and incubated at 25°C and 95–99 per cent. R.H. until the mycelia had covered the feeder strips, when a sterile dish of sawdust was placed in each jar. The dishes containing the sawdust samples were $1\frac{1}{2}$ in. in diameter, $\frac{3}{16}$ in. deep, of 0·003 in. thick aluminium foil, the bottom being perforated by numerous holes (0·001–0·010 in. in diameter) to allow the fungus to grow up into the sawdust (Plate 1, Fig. 1). (These dishes may readily be prepared by smoothing and perforating clean, unlacquered caps from milk bottles.) Each dish was labelled in waterproof Indian ink and tared and approximately 1 g of air dry sawdust (of known moisture content) was weighed out into it (Plate 1, Fig. 2). As it was considered that the composition and toxicity of the wood extractives might be affected by high temperatures, the dishes of sawdust were sterilized at room temperature by fumigation with propylene oxide, as suggested by Hansen and Snyder (1947). The dishes were placed in 4-in. petri dishes and held overnight in a gas-tight vessel containing propylene oxide (4 ml per litre of air space). They were stored for 3 days to allow any residual propylene oxide to evaporate before being placed in the culture jars (Plate 1, Fig. 3), which were then incubated at 25°C and 95–99 per cent. R.H.

Under these conditions, hyphae of the test fungi penetrated throughout the sawdust in the dish in a few days and decay proceeded rapidly in susceptible material,

even though no water or nutrient had been added to the sawdust. Apparently the fungus was able to obtain sufficient water for its needs, since the final moisture content was often as high as 80 per cent. even in relatively unattacked sawdust. The incubation period was 7 weeks for *Coniophora cerebella*, 9 weeks for *Trametes lilacino-gilva*, and 14 weeks for *Coriolus versicolor* and *Fomes durus*.

At the end of the incubation period, the dishes of sawdust were oven dried for 48 hr at 105°C and reweighed. The percentage decrease in oven dry weight of the sawdust was calculated for each sample, and the results statistically analysed to determine the significance of the differences observed. Each type of sawdust for each tree was tested in duplicate against each test fungus, except that unextracted tallowwood and untreated mountain ash were tested in quadruplicate.

TABLE 2

EXTRACTIVE CONTENT AND pH OF TALLOWWOOD SAWDUSTS

|  | Tree MI7 | Tree TWI0 | Tree TW9 | Tree QMI25 |
|---|---|---|---|---|
| Successive extractives as percentage of oven-dry weight: | | | | |
| Ether | 2·82 | 2·52 | 2·92 | 2·43 |
| Methanol | 15·05 | 13·80 | 12·77 | 12·21 |
| Acetone | 0·10 | 0·07 | 0·04 | 0·10 |
| Water | 0·81 | 1·20 | 1·06 | 0·56 |
| Alkali | 5·71 | 4·97* | 0·70 | 0·44 |
| Total, excluding alkali extractives | 18·78 | 17·59 | 16·79 | 15·30 |
| Total, including alkali extractives | 24·49 | 22·56* | 17·49 | 15·74 |
| pH of sawdusts: | | | | |
| Unextracted | 3·8 | 3·8 | 3·7 | 3·6 |
| Ether and methanol-extracted | 4·7 | 4·7 | 4·6 | 4·6 |
| Ether, methanol, acetone, and water-extracted | 4·8 | 5·1 | 5·1 | 5·1 |
| pH of methanol extract in water: | 3·6 | 3·6 | 3·6 | 3·4 |

\* 0·1N alkali (other results for 0·01N alkali).

### (e) *Dilution Tests with Methanol Extract in Sawdust*

Preliminary work had indicated that the material responsible for the decay resistance of tallowwood would probably be located in the methanol extract. Accordingly, this extract was investigated more intensively and was tested at a wide range of concentrations in mountain ash sawdust to determine the degree of its toxicity. These concentrations are expressed as percentages of the concentration (w/w) present in the relevant untreated tallowwood.

## (f) Dilution Tests with Methanol Extract in Agar

Agar toxicity tests were carried out using the methanol extract in concentrations (w/w) corresponding to those in sawdust. The test was carried out in small glass phials each containing a total of 2 g of solid medium (Plate 1, Fig. 4). The medium chosen for this purpose contained 2 per cent. agar, 4 per cent. carboxymethylcellulose, and 2 per cent. malt extract, in tap water. The methanol extract, agar, and carboxymethylcellulose were mixed in the solid state as fine powders in the phial, the correct volume of 2 per cent. malt extract solution in tap water added, and the mixture stirred. After steaming for 25 min to dissolve the agar, the viscous material was stirred, and the phial capped and laid flat so that the agar solidified as a layer along one side. The phials were then sterilized with propylene oxide in the same manner as the sawdust dishes used in the decay tests. The phials were inoculated at one end of the agar strip, and incubated at 25°C and 95–99 per cent. R.H. for several weeks. Once growth had commenced, the position of the advancing front of mycelium growing along the phial was recorded at 2–3 day intervals and the average rate of linear growth calculated as a percentage of control growth.

## III. Results

### (a) Chemical Results

Tallowwood is so named because of its greasy nature and it is the material responsible for this which is extracted by ether. The product, a yellow viscous oil, was readily extracted and was present in 2–3 per cent. yield, based on the oven dry weight (Table 2). This probably included the steroid from which *cyclo*eucalenol was obtained (Cox, King, and King 1956). Concentration of the methanol extract from the ether-extracted tallowwood readily gave a brown solid. The yield from different trees varied between 12 and 15 per cent. Ether, methanol-extracted tallowwood had lost much of its original lustre, but the original colour variations between trees could still be noticed. Acetone extraction of ether, methanol-extracted tallowwood removed a very small amount of a grey-brown solid which was, in all probability, related to the methanol extract. Water extraction of ether, methanol, acetone-extracted tallowwood also removed a comparatively small amount of grey-brown solid. At this stage it was still possible to discern colour differences between trees MI7 and QMI25, on the one hand, and trees TW10 and TW9 on the other. Colour differences between MI7 and QMI25, or between TW10 and TW9, had disappeared, the two former were buff and the two latter grey.

Whilst it is unlikely that ether or methanol extraction of tallowwood had much effect on the wood structure, hot water extraction would cause a small amount of hydrolysis, which may have slightly lowered the resistance of the wood to decay. The final extraction with weak alkali would unquestionably have affected the cell wall as well as the extraneous materials present and probably predisposed the wood to fungal attack.

Tallowwood had a pH of 3·6–3·8 and gave a methanol extract with a pH of 3·4–3·8 in aqueous medium, the ether, methanol-extracted tallowwood having a pH of 4·6–4·7. After the aqueous extraction the pH rose to 4·9–5·1.

TABLE 3

PERCENTAGE WEIGHT LOSSES OF TALLOWWOOD SAWDUSTS

Values given are mean weight losses for two replicate specimens, except values for untreated tallowwood and untreated mountain ash which are means of four replicates

| Test Fungus | Tree No. | Untreated Tallowwood Sawdust | Ether (1) | (1) followed by Methanol (2) | (1), (2) followed by Acetone (3) | (1), (2), (3) followed by Water (4) | (1), (2), (3), (4) followed by Alkali (5) | Untreated Sawdust | Alkali-extracted Sawdust |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Tallowwood Sawdust after Successive Extractions with: | | | | Mountain Ash Controls | |
| Trametes lilacino-gilva DFP 1109 | MI7 | 0·7 | 0·2 | 11·5 | 23·4 | 29·0 | 19·8 | | |
| | TW10 | 0·8 | 0·0 | 14·4 | 17·3 | 30·0 | 34·4 | | |
| | TW9 | 0·5 | 0·4 | 9·4 | 29·4 | 30·8 | 35·4 | | |
| | QMI25 | 0·6 | 0·6 | 24·8 | 33·0 | 34·8 | 25·4 | | |
| Mean* | | 0·7 | 0·3 | 15·0 | 25·8 | 31·1 | 28·8 | 43·8 | 44·8 |
| (signs) | | = | ≪ | ≪ | < | = | = | | |
| Coniophora cerebella DFP 1779 | MI7 | 0·0 | 0·0 | 44·4 | 40·0 | 41·6 | 42·6 | | |
| | TW10 | -0·6 | 0·2 | 47·2 | 44·1 | 49·4 | 57·4 | | |
| | TW9 | 1·2 | -0·2 | 44·6 | 45·0 | 46·2 | 52·2 | | |
| | QMI25 | -1·4 | 0·2 | 47·5 | 47·4 | 49·6 | 50·8 | | |
| Mean | | -0·8 | 0·1 | 46·0 | 44·1 | 46·7 | 50·8 | 50·6 | 60·8 |
| (signs) | | = | ≪ | ≪ | = | = | = | | |
| Coriolus versicolor DFP 2666 | MI7 | 1·0 | -0·4 | 22·0 | 15·6 | 29·8 | 26·2 | | |
| | TW10 | -0·2 | 0·0 | 19·8 | 27·0 | 22·3 | 32·2 | | |
| | TW9 | -0·4 | 2·8 | 21·4 | 28·9 | 20·0 | 32·6 | | |
| | QMI25 | -1·0 | 1·0 | 25·0 | 22·0 | 24·0 | 29·0 | | |
| Mean | | -0·2 | 0·9 | 22·0 | 23·4 | 24·0 | 30·0 | 25·8 | 40·4 |
| (signs) | | = | ≪ | ≪ | = | = | = | | |
| Fomes durus DFP 3882 | MI7 | 0·5 | -2·7 | 15·5 | 20·0 | 18·4 | 16·7 | | |
| | TW10 | -4·4 | -3·9 | 16·6 | 11·4 | 20·0 | 23·4 | | |
| | TW9 | -0·9 | -1·4 | 20·7 | 15·2 | 4·8 | 18·2 | | |
| | QMI25 | -1·2 | -3·8 | 23·4 | 10·3 | 17·8 | 12·8 | | |
| Mean | | -1·5 | -3·0 | 19·1 | 14·2 | 15·2 | 17·8 | 39·2 | 26·0 |
| (signs) | | = | ≪ | ≪ | = | = | = | | |

* Means linked by the signs = do not differ significantly, < differ significantly at 5 per cent. level, and ≪ at 1 per cent. level.

### (b) *Decay Resistance of Untreated Tallowwood*

Even when reduced to fine sawdust, tallowwood proved very resistant to decay, virtually no loss in weight being caused by any of the four test fungi (Table 3). Other tests were made with longer incubation periods, but even after 30 weeks'

TABLE 4

PERCENTAGE WEIGHT LOSSES OF MOUNTAIN ASH SAWDUSTS CONTAINING TALLOWWOOD
EXTRACTIVES

Values given are mean weight losses for two replicate specimens, except values for untreated controls which are means of four replicates

| Test Fungus | Tree No. | Source of Extractives | | | | | Untreated Control |
|---|---|---|---|---|---|---|---|
| | | Ether Extract | Methanol Extract | Acetone Extract | Water Extract | Alkali Extract | |
| *Trametes lilacino-gilva* | MI7 | 40·0 | 1·4 | 38·6 | 40·2 | 9·2 | |
| DFP 1109 | TW10 | 45·5 | 1·0 | 26·2 | 42·8 | — | |
| | TW9 | 45·2 | 2·0 | 33·4 | 41·0 | 35·4 | |
| | QMI25 | 43·3 | 1·6 | 42·6 | 32·0 | 26·6 | |
| Mean | | 44·0† | 1·5** | 35·2* | 39·0* | 23·8** | 43·8 |
| *Coniophora cerebella* | MI7 | 49·2 | 1·1 | 51·8 | 51·5 | 52·0 | |
| DFP 1779 | TW10 | 48·3 | 0·9 | 48·0 | 47·4 | — | |
| | TW9 | 50·2 | 1·1 | 50·2 | 47·2 | 48·4 | |
| | QMI25 | 48·4 | 0·4 | 52·8 | 47·8 | 49·0 | |
| Mean | | 49·0† | 0·9** | 50·7† | 48·5† | 49·8† | 50·6 |
| *Coriolus versicolor* | MI7 | 41·9 | 1·0 | 31·9 | 31·8 | 35·4 | |
| DFP 2666 | TW10 | 40·0 | 0·3 | 36·7 | 20·2 | — | |
| | TW9 | 30·4 | −0·4 | 44·2 | 37·0 | 35·6 | |
| | QMI25 | 26·8 | 0·6 | 38·2 | 26·1 | 38·0 | |
| Mean | | 34·7† | 0·4** | 37·7* | 28·8† | 36·3* | 25·8 |
| *Fomes durus* | MI7 | 33·2 | −1·4 | 42·8 | 22·6 | 30·1 | |
| DFP 3882 | TW10 | 40·2 | −3·6 | 37·6 | 25·6 | — | |
| | TW9 | 39·2 | −1·4 | 40·0 | 21·2 | — | |
| | QMI25 | 15·8 | −0·4 | 39·3 | 14·4 | 36·1 | |
| Mean | | 32·1† | −1·7** | 40·0† | 21·0** | 33·1† | 39·2 |

\* Differs significantly from control at 5 per cent. level.
\*\* Differs significantly from control at 1 per cent. level.
† Does not differ significantly from control.

incubation, weight losses were negligible except with *Fomes durus*, which produced weight losses of up to 25 per cent. There was no appreciable variation among trees in regard to decay resistance, either in the main test or in the longer tests.

## (c) Decay Resistance of Extracted Tallowwood

The amounts of decay occurring in unextracted tallowwood and in tallowwood subjected to the various extraction procedures are set out in Tables 3 and 5 and the amounts of decay occurring in untreated mountain ash and in alkali-extracted mountain ash are also given for comparison. It will be seen that in all cases the methanol extraction produced a highly significant loss of decay resistance, whereas no other extraction procedure had any consistent effect. Even after extraction with ether, methanol, acetone, and water, the tallowwood sawdust was significantly (1 per cent. level) more resistant than unextracted mountain ash sawdust to attack by *Trametes lilacino-gilva* and *Fomes durus*, though not to attack by *Coniophora*

TABLE 5

PERCENTAGE WEIGHT LOSSES OF TALLOWWOOD SAWDUSTS AND OF MOUNTAIN ASH SAWDUSTS CONTAINING TALLOWWOOD EXTRACTIVES AFTER VACUUM DRYING

Values given are mean weight losses for two replicate specimens

| Test Fungus | Tree No. | Tallowwood Sawdust after Successive Extractions with: | | | Mountain Ash Sawdust | |
|---|---|---|---|---|---|---|
| | | Ether followed by Methanol (1) | (1) followed by Acetone (2) | (1), (2) followed by Water (3) | Untreated | Containing Acetone Extract |
| *Trametes lilacino-* | MI7 | 25·4 | 25·6 | 29·8 | | 43·2 |
| *gilva* | TW10 | 29·0 | 29·8 | 36·8 | | 45·9 |
| DFP 1109 | TW9 | 29·6 | 33·8 | 35·0 | | 44·2 |
| | QMI25 | 29·8 | 32·8 | 39·5 | | 41·8 |
| Mean* | | 28·4    = | 30·5    < | 35·3    ≪ | 45·4    = | 43·8 |

* Means linked by the sign = do not differ significantly, < differ significantly at 5 per cent. level, ≪ differ significantly at 1 per cent. level.

*cerebella* and *Coriolus versicolor*. After further extraction with dilute alkali, the sawdust was still significantly more resistant than alkali-extracted mountain ash, except in the case of *Fomes durus*, but owing to the small number of alkali-extracted mountain ash controls involved in this comparison, too much importance should not be attached to this difference.

## (d) Toxicity of Tallowwood Extractives

The effects of the various tallowwood extracts in protecting mountain ash sawdust against decay are shown in Tables 4 and 5. These show that the methanol extract was highly toxic in all cases, and that no other extract produced a consistently toxic effect, although some showed slight toxicity to one or more fungi.

The relative effectiveness of different concentrations of this toxic methanol extract in mountain ash sawdust is shown in Figure 1. Dosage–response curves for three of the fungi are closely similar, substantially complete inhibition being obtained only when the concentration approaches that in the original tallowwood, but *Coriolus versicolor* was strongly inhibited by low concentrations of the extract.
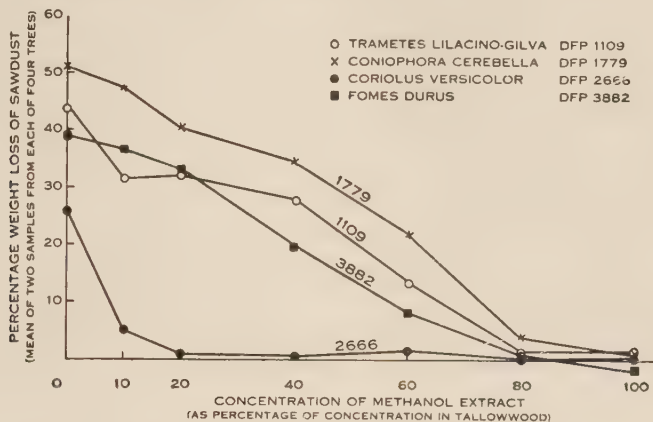


Fig. 1.—Effect of increasing concentrations of methanol extractives on decay of mountain ash sawdust.

The toxicity of the methanol extract in agar is shown in Figure 2. (The growth of *Fomes durus* on agar was extremely erratic and no reproducible results were obtained with this fungus.) Results are reasonably comparable with those of the sawdust bioassay, but the methanol extract is apparently less toxic in agar than in wood.
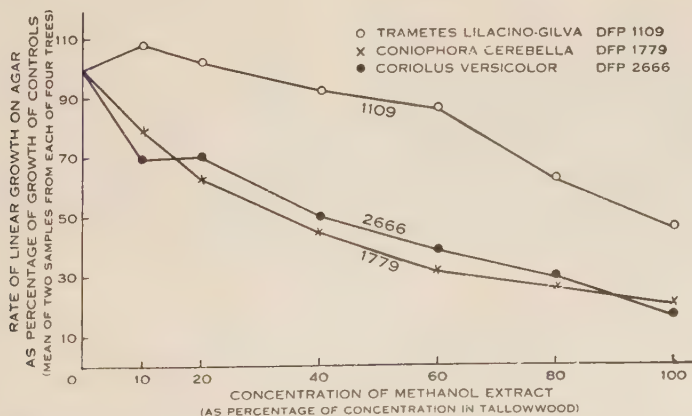


Fig. 2.—Effect of increasing concentrations of methanol extractives on growth of test fungi on agar.

## IV. Discussion

The results obtained show that tallowwood is highly resistant to decay when tested in the form of fine sawdust, but that this resistance is largely destroyed by

the removal, during the methanol extraction stage of the extraction procedure used, of a substance (or substances) which is highly toxic to all four test fungi used. It is of interest that this methanol extract is much more toxic to *Coriolus versicolor*, which was known to find difficulty in attacking eucalypt heartwood, than to *Coniophora cerebella* and *Trametes lilacino-gilva*, which were known to attack even durable eucalypts. The data in Figure 1 suggests that tallowwood contains barely enough toxic material to protect it against these fungi, but as the distribution of the material in the impregnated mountain ash sawdust will be different from that in the tallowwood, the "margin of safety" may be greater than these data suggest.

The relatively slight difference between the decay resistance of tallowwood extracted successively with ether, methanol, acetone, and water and that of the non-durable mountain ash indicates that only a small proportion of the decay resistance of tallowwood can be due to the chemical composition or fine structure of its cell walls. The extent to which the decay resistance of tallowwood is enhanced by its anatomical structure, and particularly by its low void volume, cannot be clearly evaluated here since the effect of these factors was partly, but not entirely, removed by converting the heartwood to fine sawdust. The fact that this fine sawdust was still highly resistant to decay suggests that the effect of gross anatomical structure is not very important in this species.

There are some indications in Tables 3 and 4 that extractives other than those removed by the methanol play a minor part. However, close inspection of the detailed results, particularly those for tallowwood exposed to *Trametes lilacino-gilva* after methanol and after acetone extraction, suggested that some of these indications might be due to retention of toxic amounts of solvent in the sawdust. A portion of the test was therefore repeated for this fungus, the procedure being as before except that all sawdust was vacuum dried. The results, as shown in Table 5, differ from those of the main test in that the acetone extract showed no toxicity and the effect of extraction with acetone was not significant. It seems likely, therefore, that effects connected with the acetone extraction may be due to retention of acetone by the sawdust in amounts large enough to affect the growth of some fungi. Some organic solvents are very strongly retained by sawdust and cellulose even at temperatures well above their boiling point, e.g. methanol, acetone, ethanol–benzene (Wiertelak and Garbaczowna 1935; Anderson 1946).

It might be inferred from Tables 3 and 4 that the toxic extractives of tallowwood are readily soluble in methanol but not in ether. Some caution in accepting this is indicated by Erdtman's "membrane substances" (Erdtman 1943; Kondo and Kitamura 1955a, 1955b; Kondo, Koyama, and Tanaka 1955) which prevent the extraction with ether of the ether-soluble pinosylvins in pine heartwood; both membrane substances and pinosylvin are readily extracted with methanol or acetone. Work here has shown that a similar situation occurs with some of the ether solubles in tallowwood. Chromatographic fractionation of the methanol extract is now being carried out, together with estimation of the toxicity of the various fractions.

## THE CAUSES OF NATURAL DURABILITY IN TIMBER. I
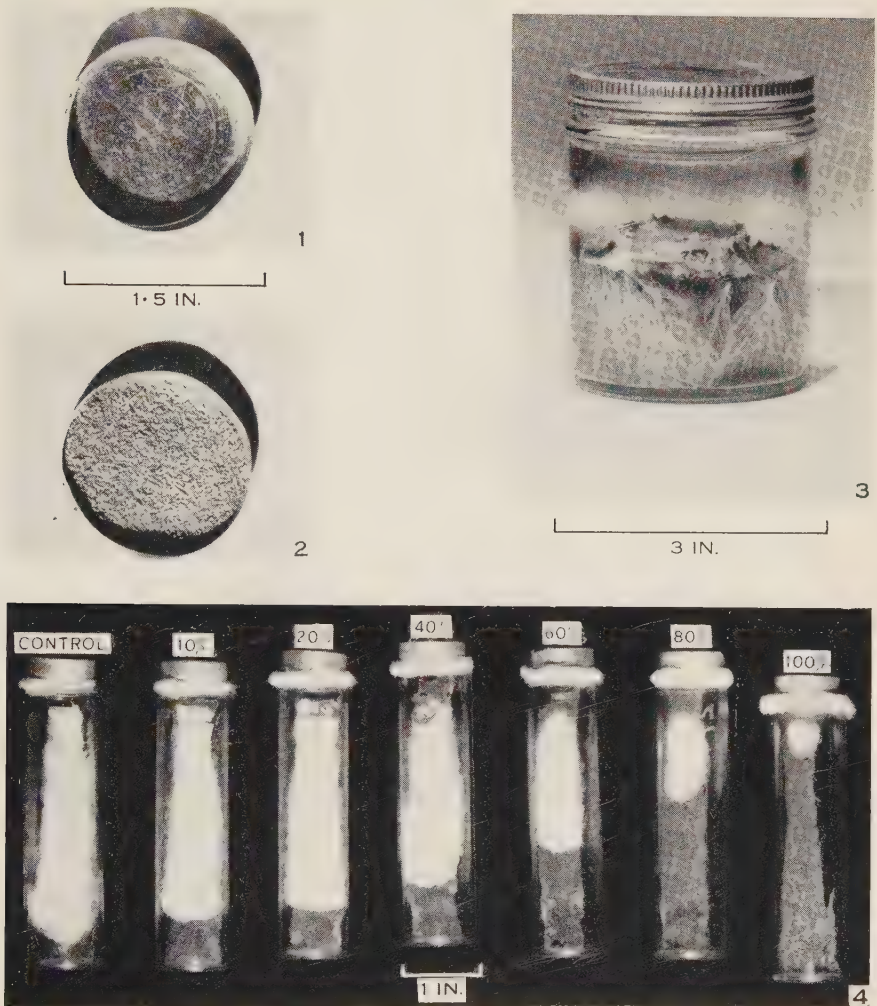


Fig. 1.—Perforated aluminium dish, empty.

Fig. 2.—Perforated aluminium dish, with 1 g sawdust.

Fig. 3.—Culture jar, with dish of sawdust, after incubation.

Fig. 4.—Effect of increasing concentrations of methanol extract in nutrient agar on growth of test fungus.

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# A HYPOTHESIS OF DEVELOPMENTAL SELECTION EXEMPLIFIED BY LETHAL AND SEMI-LETHAL MUTANTS OF *ARABIDOPSIS*

By J. Langridge*

*Summary*

A hypothesis of developmental selection is presented in which the internal factors of selection, barriers imposed by ontogenetic development, are considered in relation to the degree of survival of newly arisen deleterious mutations. This hypothesis states that the survival of such mutant genes to the stage of germination depends on the diffusibility of the metabolite required by the mutants and the ontogenetic stage at which it is required. As a corollary of the hypothesis, those lethal mutants of flowering plants that appear after germination should, with certain predictable exceptions, have requirements for low molecular weight substances which may be supplied from external sources.

As an experimental test of the hypothesis, the requirements for normal growth of 11 lethal or semi-lethal mutants of *Arabidopsis thaliana* (L.) Heynh. have been examined. The six "reparable" mutants require the following: (1) thiamin; (2) choline; (3) coconut milk; (4) sucrose or glucose; (5) a high osmotic pressure; (6) an alternation of temperatures for flowering. Of the five "irreparable" mutants, (1) has decreased embryo growth; (2) lacks cotyledons; (3) lacks chlorophyll; (4) and (5) lack chloroplasts. It is considered that the behaviour of these mutants is generally in accord with the hypothesis.

## I. Introduction

Lethal mutants, although frequently appearing in flowering plants, have been regarded with little interest, and with the exception of chlorophyll-deficient mutants, no systematic examination of their frequency of occurrence or physiology has been made. The reason for this is that precise work on the organic nutrition of intact flowering plants is technically difficult. In contrast with the majority of micro-organisms, higher plants are diploid and thus the isolation of mutants is laborious, their large-scale aseptic culture is seldom practicable, and their average life cycle is generally a matter of months. However, it has recently been shown (Langridge 1955) that biochemically deficient mutants of the *Neurospora* type may be isolated and characterized in the crucifer, *Arabidopsis thaliana* (L.) Heynh. The examination of the first 11 lethals or semi-lethals isolated in this plant has now been completed, and a summary of their behaviour is presented below as an experimental test of a hypothesis of developmental selection.

This hypothesis states that the selection against deleterious mutations during the ontogenetic development of a flowering plant is determined (i) by the time of action of the gene concerned, and (ii) by the diffusibility of its metabolically active product.

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It is expected that selection of this nature will be most pronounced in flowering plants. Animals have only limited capacity for synthesis of essential substances in the low molecular weight range, so that there is little possibility of deleterious mutations being maintained by synergism. Such mutations will normally cause the early death of the embryo. In most microorganisms, on the other hand, a relative lack of pre-germinal differentiation excludes rigorous selection against newly arisen mutations. When the mature conidia of a fungus such as *Neurospora* are irradiated and immediately placed on a nutrient medium, the first possibly gene-controlled developmental process to occur is that of germination. No developmental selection is involved, and therefore, only about 7 per cent. of non- or slow-growing mutants have synthesis deficiencies which can be made good with *Neurospora* autolysate (Lein, Mitchell, and Houlahan 1948).

However, when the seeds or pollen grains of angiosperms are irradiated, any induced mutation may be subject to elimination by any one of a series of developmental barriers before it can be expressed as a homozygous sporophytic character. This selection against certain mutations by the sieves of gametophyte, endosperm, and embryo development is important when considering the types of biochemical mutants to be expected in flowering plants.

It will be shown below that X-irradiation of the seeds of *A. thaliana* may cause a mutation in one cell of a usually two-celled apical meristem. This mutation, except when it is a dominant inhibitor of an essential cellular function, will be maintained as a chimera in the $X_1$ plant both by its wild-type allele in the same cell and by the normal tissue of the remainder of the plant. The mutant allele will thus remain shielded from selection until it becomes separated from its wild-type allele at meiosis. Without considering the details of selection during the critical part of the plant's life cycle between meiosis and germination, one may predict that there will be two categories of mutants with respect to this selection.

### (a) *Mutations Escaping Pre-germinal Developmental Selection*

There is a class of genes which, although normally active throughout the whole of the plant's life cycle, show a mutant phenotype during only part of this cycle. This device lowers the deleterious effect of a particular mutation. In plants carrying the mutant allele *wx*, for example, only amylopectin is formed in the pollen grain, female gametophyte, and endosperm instead of both amylose and amylopectin (Sprague, Brimhall, and Hixon 1943). However, both types of starch are found in the post-germination sporophyte. Another example is the gene *Y* (yellow endosperm) in maize. The mutant lacks carotenoids in the endosperm (Mangelsdorf and Fraps 1931), yet, since recessive plants grow normally, carotenoids must be present in large amounts in the leaves, where they are probably essential for the maintenance of chlorophyll (Griffith *et al.* 1955). It is an open question as to whether there are separate genes acting sequentially to perform two apparently identical functions, or whether a genetic block in one tissue is not necessarily a block in another tissue differently differentiated.

Certain evidence suggests that some mutant genes may escape elimination because they are mutations of genes which are inactive in the seed. The embryo of

the pea, for example, has a complete set of genes for the formation of nicotinic acid, yet it is unable to carry out this synthesis (Bonner 1938), although it is improbable that a light-catalyzed reaction is involved. The young pea plant may acquire the ability to form nicotinic acid adaptively, for one would expect an extensive synthesis of adaptive enzymes at or soon after germination, when the organism is exposed to a new set of environmental stimuli. *Arabidopsis* embryos are unable to utilize nitrate (Rijven, unpublished data), yet the seedling can do so, presumably because nitrate reductase is then formed. The requirements of embryos for other organic compounds may also be a reflection of failure of the genes to act at this stage.

A third class of gene effect also is not subject to selection during seed growth because the product of the biosynthetic chain in which the gene acts is not required before germination. Mutational blocks in photosynthesis are of this type, the most common being those affecting the formation of chlorophyll and plastids.

A final group of genes whose functions may be lost by mutation, although the mutated gene does not cause the death of the seed, consists of those genes active in the synthesis of diffusible substances. In these cases it is expected that the deficiency will be made good by diffusion of the required substance from the maternal plant.

### (b) *Mutations Eliminated by Pre-germinal Development*

It seems probable that those mutations which lead to inviable gametophytes or seeds and thus are not expressed in the growing plant, may be of three types with respect to function.

The largest class will consist of mutations affecting the synthesis of high molecular weight materials which cannot diffuse from one genetic type of tissue to another. Included here are mutations blocking the formation of polypeptides and proteins, many phosphorylated compounds, conjugated vitamins, and nucleic acids.

Another type of mutation that will be eliminated is concerned with the metabolism of the resting seed or catabolic reactions of the germination process. Mutations of the genes active in the Krebs cycle, for example, may survive until the seed is mature, but thereafter they will be lethal and germination will fail.

Finally, it is doubtful if gene mutations which cause a multiple loss of essential metabolites will survive to germination, even if the metabolites are diffusible. This class includes genes acting early in biosynthetic pathways such as amino acid synthesis (Davis 1951), and genes controlling relatively non-specific enzymes (e.g. Rudman and Meister 1953).

The conclusion to be drawn from this discussion is that, subject to the restrictions outlined above, most of the non-growing mutants of a flowering plant should be lacking in the ability to form diffusible substances and, therefore, they should be capable of responding to supplements.

## II. METHODS

A description of the experimental plant, *A. thaliana*, and of the method used for its aseptic culture has been published previously (Langridge 1957).

### (a) Production of Mutations

Mutations were induced by X-irradiation of seeds of the race Estland at the dosage recommended by Reinholz (1947). Seeds soaked for 40 hr received 6000 r at 316 r/min, 130 kV, 20 mA, and with a half-value layer of 2.9 mm of aluminium. The seeds were germinated on filter paper, the seedlings transferred to pots of steam-sterilized soil, and the $X_1$ plants grown in the glass-house.

For the isolation of mutants, all seeds from an $X_1$ plant were harvested, mixed thoroughly by shaking, and 16 seeds taken at random were sown separately in test tubes containing sterile mineral agar. When the $X_2$ segregation ratio is 7 : 1 (see below), a family size of 16 is only about 90 per cent. efficient in detecting mutants. However, by this isolation method each seed may be accounted for, so that all lethal and semi-lethal segregants have been detected, although many of the minor morphological mutants may have been missed. When an $X_1$ plant was found to be heterozygous for a mutation, further seeds were usually sown to determine the $X_2$ segregation ratios shown in Tables 4, 5, and 6.

### (b) Supplements

The following supplements are the standard ones used to determine the requirements of growth mutants. None of them increases the dry weight of wild-type plants growing at normal temperatures. All extracts, hydrolyzates, or solutions are sterilized by filtration through sintered-glass filters (Gallenkamp, porosity 5), and the aseptic liquids are transferred to heat-sterilized vaccine bottles for storage. When required, aliquots are taken from the bottles using an alcohol-sterilized hypodermic syringe and added to autoclaved agar–mineral medium before gelling has occurred. If the mutant shows a response to amino acid or vitamin solutions, absence of interaction is initially assumed and the specific compound required is determined by means of the screening procedure recommended by Lindegren and Lindegren (1951).

(i) *Coconut Milk.*—Milk from green coconuts was brought to pH 3.0, extracted four times with freshly distilled ether, and adjusted to pH 6.0. Residual ether was removed by heating the milk on a water-bath at 35°C. It is used at the rate of 0.4 ml per plant—i.e. per 5 ml medium.

(ii) *Pea Seed Diffusate.*—This was prepared according to the method of Bonner, Haagen-Smit, and Went (1939). Five diffusate fractions were obtained by soaking sterilized seeds for 24 hr in successive volumes of sterile distilled water. The first fraction was discarded, and the remaining ones were combined and used without concentration. Only 0.25 ml could be supplied to each plant, as higher concentrations delayed germination by about 6 days and strongly inhibited root growth.

(iii) *Nucleic Acid Hydrolysate.*—Sodium nucleate (from yeast) was dissolved in 0.1N NaOH, hydrolysed by heating for 30 min at 50°C, neutralized, and made up to the required volume. It was used at the rate of 0.4 mg nucleic acid derivatives per plant.

(iv) *Vitamins.*—The relative amounts of the different water-soluble vitamins were based on published figures for their occurrence in plant tissues (Cheldelin and

Williams 1942; Schopfer 1949). The concentrations in the stock solution were adjusted so that an aliquot of 0.1 ml would contain the amounts of vitamins normally present in a plant of 20 mg dry weight (Table 1).

TABLE 1

VITAMIN SOLUTION

| Vitamin | Amount Supplied per Plant ($\mu$g) | Vitamin | Amount Supplied per Plant ($\mu$g) |
|---|---|---|---|
| Thiamin hydrochloride | 0·2 | $i$-Inositol | 40·0 |
| Riboflavin | 0·2 | Folic acid | 0·2 |
| Nicotinic acid | 1·0 | Ascorbic acid | 4·0 |
| D-Pantothenate, calcium salt | 0·2 | Choline chloride | 0·2 |
| Pyridoxine hydrochloride | 0·2 | $p$-Aminobenzoic acid | 0·2 |
| D-Biotin | 0·01 | | |

(v) *Amino Acids.*—The amino acid solution contained individual amino acids in the proportions found in the plant protein edestin (Tristram 1949). However, the plants are very sensitive to amino acids, tolerating only about 1/20 of the calculated optimal supplement when a complete mixture is used. Therefore, the amino acids were grouped according to structural relationships, the tolerance level determined for each group, and solutions of these were usually used in preference to the complete mixture (Table 2).

TABLE 2

AMINO ACID SOLUTIONS

| Solution No. | Amino Acid | Amount Supplied per Plant ($\mu$g) | Solution No. | Amino Acid | Amount Supplied per Plant ($\mu$g) |
|---|---|---|---|---|---|
| I | Glycine | 7 | IV | L-Cystine | 12 |
| | $\beta$-Alanine | 82 | | L-Cysteine | 12 |
| | DL-Serine | 25 | | L-Methionine | 26 |
| | L-Threonine | 52 | | L-Histidine | 63 |
| | | | | L-Proline | 54 |
| II | L-Valine | 52 | | | |
| | L-Leucine | 50 | V | L-Aspartic acid | 24 |
| | L-Isoleucine | 61 | | L-Glutamic acid | 34 |
| III | L-Phenylalanine | 10 | VI | L-Lysine | 25 |
| | L-Tyrosine | 10 | | L-Arginine | 54 |
| | L-Tryptophan | 7 | | | |

III. RESULTS

(a) *The $X_1$ Generation*

About 50 per cent. of the X-irradiated seeds germinated at the same time as unirradiated ones, the remainder having delayed germination or none at all (Table 3).

Most of the seedlings from seeds with definitely delayed germination had poorly developed cotyledons and failed to grow on planting into soil. Many others, whose seeds were only about 1 day later than the controls in germinating, produced only a few leaves which were pale green and of a smooth, round, drooping appearance. They became yellow and died within 3 weeks of planting out.

During growth, many of the plants showed the non-inherited morphological abnormalities characteristic of high-dosage X-ray treatment. These abnormalities included split leaves, multiple and fused flower stalks, fused leaves, single flowers at the leaf axils, and death of the main floral primordium. The first three leaves of one plant contained a chlorophyll-deficient sector, but its progeny were of a normal green colour. No dominant mutations were seen.

TABLE 3

GERMINATION AND SURVIVAL AFTER IRRADIATION

| Race | No. of Seeds Irradiated | Germination (%) | Plants Surviving and Producing Seed (%) |
|---|---|---|---|
| Estland (control) | 100 | 87 | 72 |
| Estland (irradiated) | 600 | 80 | 21 |
| Graz (irradiated) | 750 | 69 | 8 |
| Enkheim (irradiated) | 600 | 73 | 16 |

### (b) The $X_2$ Generation

The irradiation of seeds causes mutations as a result of ionization in a multicellular apical meristem. Consequently, the $X_1$ plants will be chimerical for any mutations so induced.

From segregation data, allowance may be made for the chimerical state and the mutation rate per primordial cell calculated as follows. The mean segregation ratio of normal to mutant for 15 mutations, obtained from sowing mixed seeds from $X_1$ plants, was 8 : 1. This segregation ratio approximates to the 7 : 1 expected if the mean number of primordial cells, which eventually form flowering shoots bearing seed, is two at the time of irradiation. Then, assuming two primordial cells per plant, both of which are sampled yielding a 7 : 1 ratio in families of 16 plants, the apparent mutation rate will be $2\chi (1-(0.875)^{16})$, i.e. $1.76\chi$, where $\chi$ is the mutation rate per primordial cell.

With a dose of 6000 r, the apparent mutation rate, based on the examination of 112 $X_2$ families, was 21 per cent. Thus the probability of any given $X_1$ plant having a detectable mutation will be 0·21 and the probability per primordial cell 0·12.

These are rather inaccurate calculations, for only 60 per cent. of the mutant families give the expected 7 : 1 ratio, the remainder having $X_2$ segregations suggestive of origin in a one-, four-, or five-celled meristem. In addition, the diagrams given

by Kaukis and Reitz (1955) indicate that, between normal and mutant tissue in a chimera, there may exist competition leading to a gradual suppression of the mutant sector.

Four sorts of abnormality occur in $X_2$ families:

(i) Single gene mutations.

(ii) Variations produced by the contemporary environment, accounting for 60–70 per cent. of the total apparent variation. Included here are fasciations of the type found in the $X_1$ generation.

(iii) Variations environmentally induced at the time of seed development, e.g. mono- and tricotyledonous seedlings.

(iv) Abnormal plants, usually with reduced fertility, which continue to segregate in successive selfed generations. When examined cytologically, these are found to be due to chromosomal deletions or rearrangements.

TABLE 4

LIST OF MORPHOLOGICAL MUTANTS

| Mutant No. | Phenotype | $X_2$ Segregation | |
| --- | --- | --- | --- |
| | | Normal | Mutant |
| 1014/12 | Pointed leaves | 14 | 1 |
| 2075/4 | Rounded leaf tips and short petioles | 38 | 3 |
| 1072/9 | Leaves two-thirds normal size | 15 | 1 |
| 1064/1 | Angular, toothed leaves | 43 | 5 |
| 1010/15 | Early flowering | 15 | 1 |
| 1036/16 | Late flowering | 15 | 1 |
| 1090/1 | White seeds | * | * |

*Segregated in $X_3$ generation.

## (c) Mutants

Twenty-four mutants were obtained from testing 112 families. Eleven of these were lethals or semi-lethals, six were chromosomal mutants, and seven were morphological mutants whose viability was not noticeably decreased. Of the mutants designated as chromosomal, only two have been examined cytologically, but mutants of this class have a characteristic appearance and a much reduced fertility. Typically, they have irregular asymmetric leaves, smaller and narrower than the wild type, often with the leaf edges curled downwards. Most of the pollen grains are aborted and the anthers indehiscent. Plants homozygous for the deficiency appear rarely or not at all.

A list of the morphological and chromosomal mutants is presented in Tables 4 and 5. The lethal and semi-lethal mutants and their $X_2$ and $F_2$ segregations are shown in Table 6. As this study is primarily concerned with the growth requirements of mutants of low viability, these mutants are described more fully. Each of these

mutants was tested for its response to the following treatments: sucrose (2 per cent.), coconut milk, vitamins, nucleic acids, amino acids, low osmotic pressure (0·5 atm) and high osmotic pressure (2·0 atm as given by 0·025M $K_2SO_4$), low tempera-ture (20°C) and high temperature (28°C).

TABLE 5

LIST OF CHROMOSOMAL MUTANTS

| Mutant No. | Phenotype | $X_2$ Segregation | |
| --- | --- | --- | --- |
| | | Normal | Mutant |
| 1024/14 | Variable expression. Leaves coarsely serrate, with leaf edges folded inwards, or of irregular sizes. Pollen shrivelled and empty, anthers indehiscent | 40 | 6 |
| 1102/11 | Sharply pointed leaves, one-half normal size. 80 per cent. aborted pollen | 14 | 1 |
| 1007/10 | Leaves curled over at ends, smaller than normal. Heterozygous for a reciprocal translocation | 14 | 2 |
| 1084/16 | Mutant with only three rosette leaves each about one-third the normal size and having a chlorotic strip down the centre. Sterile | 13 | 2 |
| 1097/3 | Light-green leaves, very narrow and pointed. Flower stalk short and weak. Sterile | 14 | 1 |
| 1046/12 | Cotyledons spatulate; leaves small, dark green and curled; late flowering. About 60 per cent. aborted pollen. Heterozygous for a chromosome deletion | 65 | 7 |

## IV. DISCUSSION

The practical implication of the hypothesis under consideration is that, with certain predictable exceptions, all non-growing mutants of flowering plants should have requirements for diffusible substances. A test of this conclusion is provided by the behaviour of the mutants described above. Of these 11 mutants, five respond to chemicals and one responds to an altered physical environment, but it has not been possible to increase the growth of the remaining five mutants. It remains to be seen if these "irreparable mutants" invalidate the hypothesis.

Mutants 1005/7 and 2079/8 appear to belong to the same class, for they both lack chloroplasts. However, they cannot utilize sugars as would be expected if only photosynthesis were inactivated by the mutations. It seems that their lethality could be due to the loss of some component such as a protein, sterol, or lipid which is necessary both for chloroplast formation and general growth. Alternatively, the chloroplasts may be required to carry out essential functions other than photosyn-thesis. The latter alternative seems the more probable, although no synthetic properties besides photosynthesis have yet been shown to be unique to the chloroplasts. However, Sisakyan (1955) reports that the formation and oxidation of fatty acids and

TABLE 6

LIST OF LETHAL AND SEMI-LETHAL MUTANTS

| Mutant No. | Phenotype | Nutritional Requirement | Segregation Ratio | |
| --- | --- | --- | --- | --- |
| | | | $X_2$ | $F_2$ |
| 1005/7 | Light yellow cotyledons and hypo-cotyl; no leaves or secondary roots. Plastids undeveloped ($1\cdot32\pm0\cdot36\,\mu$ in diameter; wild-type, $3\cdot36\pm 0\cdot41\,\mu$) | No response to supplements | 133:22 | 44:13 |
| 2079/8 | Similar to 1005/7. Lacks chloroplasts | No response to supplements | 45:3 | 86:23 |
| 2071/13 | Normal-sized chloroplasts, but no chlorophyll. One pair of small, light yellow leaves; root system normal | With glucose or sucrose in the medium the mutant produces 4–6 rosette leaves and sometimes flowers | 44:1 | 42:22* |
| 1090/10 | Cotyledons reduced to membranous chlorophyll-less structures which usually remain within the seed coat | No response to supplements | 26:9 | 27:6 |
| 1018/6 | Rosette leaves entirely chlorotic or chlorotic towards the tips; cotyledons mottled | Thiamin (1 $\mu$g/plant) completely restores growth. The pyrimidine and thiazole portions are ineffective | 27:3 | 18:7 |
| 1025/3 | Small leaves, light green with dark green veins; very slow growing. Chlorophyll content reduced to two-thirds that of wild type | Growth accelerated by sucrose or glucose, and to a slight extent by fructose | 39:2 | 33:9 |
| 1138/1 | At 28°C, plants have thin, small, light-green, curled leaves. At 23°C, only one pair of minute leaves are formed and the plant dies early | When coconut milk is supplied, leaves are much larger, wider, and not so closely curled | 58:9 | 41:10 |
| 1031/13 | Dwarf, attaining only one-third the wild type dry weight. Relative growth rates: wild type 0·26, dwarf 0·29; net assimilation rates: wild type 0·43, dwarf 0·43; mean seed weights: wild type 33 $\mu$g, dwarf 12 $\mu$g. Dwarfness, therefore, results from poor embryo growth | No response to supplements | 58:3 | 20:4 |
| 1053/11 | Mutant with temperature requirements for flowering that are not found in the wild type. Requires 28°C for flower stalk formation, followed by low temperature (20°C) for flower production | No response to supplements | 120:16 | 30:9 |

*35 seeds failed to germinate.

TABLE 6 (*Continued*)

| Mutant No. | Phenotype | Nutritional Requirement | Segregation Ratio | |
|---|---|---|---|---|
| | | | X₂ | F₂ |
| 1023/13 | At 23°C differentiation is upset and multiple apices producing small asymmetrical leaves result. At 28°C plants are small but with normal differentiation | Requires glucose, sucrose, or K₂SO₄ to increase the osmotic pressure of the medium to at least 1·5 atm | 43:6 | 57:14 |
| EST₉ | Poor growth above 27°C. Leaf expansion and secondary root growth retarded | Normal appearance and growth with 20 µg of choline per plant | Spontaneous | 35:10 |

the incorporation of phosphorus in phospholipids takes place in the chloroplasts, and it is known that even non-photosynthesizing flowering plants (parasites and saprophytes) still retain their plastids (Schürhoff 1924). If the additional activities of the chloroplasts include the formation of a non-diffusible substance that is not required before germination, the hypothesis holds. There is evidently insufficient information to make a definite decision from a consideration of these mutants.

The mutant 2071/13 lacks only chlorophyll. The chlorophyll molecule cannot be supplied to the plant, and, although sucrose does not fully restore normal growth, this is to be expected from the work of Spoehr (1942) and Rischkow and Bulanowa (1931) with similar mutants. The roots of flowering plants do not appear to be able to take up sugar at a rate sufficient to compensate for a lack of photosynthesis.

In the two remaining mutants, 1090/10 and 1031/13, it is obvious that the genetic defect expresses itself in the embryo. The first mutation affects a process dispensable in the embryo but essential to the growing plant, while the second lowers the efficiency of a process essential to the embryo but not required by the plant. Both these mutations affect genes that are time-limited in action, and thus they have escaped pre-germination elimination.

The behaviour of the limited number of mutants that has been studied conforms, in general, with what would be expected if the hypothesis were correct. Some of the mutants do not respond to supplements, and although their behaviour does not contribute to the proof of the hypothesis, their presence was expected and is explained by it. The fact is established that, in contrast with the majority of mutants of *Neurospora* (Atwood and Mukai 1953), *Aspergillus* (Pontecorvo 1953), and *Glomerella* (Markert 1952), most of the non-growing mutants of *Arabidopsis* require diffusible substances for the restoration of their growth. The flowering plants, then, by removing through developmental selection, mutants that are unsuitable for experimental work, compensate to some extent for their diploid condition and difficulties of culture. Thus the use of biochemical mutants in small, rapidly growing plants for chemical and genetic studies should be entirely practicable. ·

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# COMPETITIVE SUPPRESSION AND THE DETECTION OF MUTATIONS IN MICROBIAL POPULATIONS

By G. W. Grigg*

*Summary*

In *Neurospora*, high concentrations of auxotrophic cells suppress the growth of prototrophic ones under conditions commonly employed in back-mutation experiments. This phenomenon is discussed in relation to the quantitative detection of rare variants in populations of microbial cells.

The mechanism of the suppression effect is due to the removal of sugar from the medium by non-growing auxotrophic conidia. If enough auxotrophs are present, insufficient free sugar is left for growth of prototrophic conidia to macroscopic colony size.

Other factors which may bias back-mutation experiments are interactions between prototrophic and auxotrophic nuclei within multinucleate conidia and the proportion of dead cells in the conidial population plated. The latter is usually higher on treated than control plates. Data are presented to show that both of these factors operate to enhance the germination and growth rate of prototrophs in treated mixed populations, but not in untreated ones. They may act synergistically with, or independently of, the competitive suppression effect in biasing estimates of the proportion of rare mutants in a microbial population.

## I. INTRODUCTION

In recent years microorganisms have replaced *Drosophila* as the most commonly used organism in mutation studies. This dates from the time when suitable techniques were developed to select certain types of mutant cells from a population. Such selection methods were developed for the bacteria *Staphylococcus aureus* and *Escherichia coli* by Demerec and his co-workers (Demerec 1946; Demerec and Latarjet 1946; Witkin 1947) when mutants resistant to certain antibiotics or phage were selected by treating sensitive cells with the appropriate agent. By the use of this assay method a large number of substances, including many inorganic salts, were classed as mutagens (Demerec, Bertani, and Flint 1951).

The observation that biochemical mutants of *Neurospora crassa* sometimes acquired the ability to grow on a minimal medium was the basis of an elegant mutation assay method developed independently by Westergaard and co-workers (see Jensen *et al*. 1951) and by Giles and Lederberg (1948). Briefly this method consists of treating a washed suspension of auxotrophic cells with a mutagen, washing further if necessary, and plating on a minimal medium in which only prototrophs will grow. Equal numbers of untreated conidia are plated on a similar medium as controls. Following incubation the numbers of prototrophs are counted on the control and "treated" plates. Any significant differences are ascribed to the mutagenic activity

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of the treatment. The general assumption is made that any conidium, whether uninucleate (microconidium) or multinucleate (macroconidium), which contains a prototrophic nucleus, will produce a visible colony when plated together with a large number of auxotrophic cells on minimal medium. This will only hold if (i) auxotrophic cells do not inhibit any prototrophs which may be present; (ii) any cell having a nucleus in which back mutation has occurred will immediately germinate and grow as rapidly on minimal medium as on medium supplemented with the growth factor required by the auxotroph.

Inhibition of protrophs by auxotrophs has been shown to occur in *Esch. coli* by Ryan and Schneider (1949) in studies of reversion of a histidine-dependent strain to histidine independence and in *N. crassa* by Grigg (1952) and Stephens and Mylroie (1953). Such inhibition may be demonstrated by plating a prototrophic and an auxotrophic strain, mixed in given proportions, on minimal medium.

In this paper data on which a previous brief report was based (Grigg 1952) will be presented, together with a suggested mechanism for the suppression. The evidence for the widespread nature of the suppression phenomenon in microbial species will be reviewed and its significance discussed.

It will also be shown how the second assumption may be tested with multinucleate conidia and some data given which suggests that the assumption may not always be valid.

## II. Material and Methods

The following strains of *N. crassa* were used in these experiments:

*Prototrophs*

MACROCONIDIAL: 70007 (*col–4\**); CMS (*col–1\**, *m su^m* (Grigg, unpublished data)).

MICROCONIDIAL: CM (*col–1*, *m\**).

*Auxotrophs*

MACROCONIDIAL: W40 (*ad–3*, *col–4\** (Kølmark and Westergaard 1949)); 37401 (*inos*); K26·9 (*hist* (Mathieson and Catcheside 1955)).

MICROCONIDIAL: 9a91 (*ad* (accumulates purple pigment), *col–1*, *m*); K51 (*meth*, *col–1*, *m*); K42 (*leuc*, *col–1*, *m*); K26 (*hist*, *col–1*, *m*); K13·5† (*meth*, *col–1*, *m*); K9† (*aden*, *col–1*, *m*); 39401–55 (*nt*, *col–1*, *m†*).

Microconidia first appear on *col–1*, *m* cultures after incubation at 25°C for 7 days (Barratt and Garnjobst 1949). Conidial production continues for about 5 days, but these conidia are very short-lived. It was our experience that higher numbers of viable conidia were obtained if young cultures were used. Macroconidia, which survive for considerably longer periods, were obtained from 4–8-day-old cultures.

The conidia were suspended in sterile distilled water and the suspensions filtered through cotton wool to remove mycelial fragments. They were then centrifuged and resuspended twice in 10-ml quantities of sterile water to free the suspension from growth factors leached from the culture medium. The density of washed conidia

*Barratt *et al.* (1954). The symbol *m* is equivalent to *pe^m* of Barratt *et al.*

†Isolated in the Botany Laboratory, Cambridge University. Linkage relations unknown.

in the stock suspension was determined by direct count in a haemocytometer and subsequently the stock suspension was diluted to the appropriate concentration. When it was necessary to determine the concentration of viable conidia, 1-ml aliquots of the appropriate dilution were plated on a medium which supported their growth.

Before sampling cell suspensions either for subsequent dilution or plating, they were vigorously shaken in 1-oz McCartney bottles. Alternate sucking and blowing from a pasteur pipette was insufficient to disperse the clumps of conidia which formed readily if a suspension were left to stand. This is illustrated in Table 1 which compares the effects of the two methods of agitation on the number of colonies which appear following the plating of equal aliquots of two dilutions series.

TABLE 1

PLATING ERRORS DUE TO INSUFFICIENT AGITATION OF SUSPENSIONS PRIOR TO SAMPLING

Number of colonies per plate after incubation for 5 days

| Pretreatment | Dilution | | | |
| --- | --- | --- | --- | --- |
| | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| Mild agitation by pipette | $41 \cdot 5 \pm 1 \cdot 9$ | $12 \cdot 5 \pm 2 \cdot 4$ | $5 \cdot 3$ | $1 \cdot 0$ |
| Vigorous mechanical agitation* | $132 \cdot 4 \pm 5 \cdot 0$ | $12 \cdot 3 \pm 1 \cdot 2$ | | |

*A sample of different cell concentration from that used in the other treatment group.

*Reconstruction experiments.*—One-ml aliquots of a dilute suspension of prototrophic conidia containing about 35 viable cells were added to tubes containing 10 ml of melted minimal agar at 40°C and also to tubes of similar medium to which 1 ml of a dense suspension of auxotropic conidia had been added. The aliquots of melted agar medium plus conidia were then poured rapidly into petri plates and incubated at 25°C in a humidified incubator. Usually there were four but sometimes three or five replicates in each group. Colony counts were made 3, 4, and 5 days after plating.

The reconstruction experiments were made on Westergaard and Mitchell's (1947) minimal medium but at pH 5·6 instead of pH 6·5 and containing either 2 per cent. glucose or 0·2 per cent. sucrose plus 0·4 per cent. sorbose as the carbohydrate source. The complete medium used was the conidiating medium of Horowitz (1947).

### III. RESULTS

#### (a) Reconstruction Experiments

All of the auxotrophic strains tested, including the strains W40 and 37401 which have been used extensively in mutation experiments (Giles and Lederberg 1948; Dickey, Cleland, and Lotz 1949; Jensen, Kølmark, and Westergaard 1949; Kølmark and Westergaard 1949; Jensen *et al.* 1951; Smith and Srb 1951), inhibited prototrophs.

### TABLE 2

SUMMARY OF RESULTS OF RECONSTRUCTION EXPERIMENTS

| Auxotrophic Strain | Prototrophic Strain | Medium* | No. of + Colonies per Plate following Plating of: | | No. of Viable Auxotrophic Conidia per Plate ($\times 10^5$) |
|---|---|---|---|---|---|
| | | | +Conidia | +and Auxotrophic Conidia | |
| W40 | CM | 2% glucose | 28·6±3·0 | 38·6±3·5† | 230 |
| W40 | CMS | 2% glucose liquid | 46·5±4·4 | 0·3 | 2700 |
| W40 | CMS | Sorbose-sucrose | Heavy growth | No growth | 150 |
| W40 | CMS | Sorbose-sucrose | 52·0±3·2 | 1·7 | 31 |
| W40 | CMS | Sorbose-sucrose | 52·0±3·2 | 10·3±3·0 | 3·1 |
| W40 | 70007 | Sorbose-sucrose | 127·5±6·9 | 138·8±10·7† | 320 |
| W40 | | | 27·0±3·0 | 22·0±0·9† | 250 |
| 37401 | CM | Sorbose-sucrose | 167·0±9·8 | 0 | 380 |
| 37401 | 70007 | Sorbose-sucrose | 160·0±3·9 | 0 | 820 |
| 37401 | | | 54·8±3·6 | 4·3±3·6 | 400 |
| K26-9 | CM | Conidiating, complete | 150·5±5·7 | 5·7±0·7 | 200 |
| K26-9 | CM | 2% glucose | 500 | 205±16·5 | 170 |
| K26-9 | CM | Sorbose-sucrose | 20·2±2·3 | 0 | 20 |
| 9a91 | CM | Sorbose-sucrose | 55·0±6·6 | 6·5±0·5 | 37·5 |
| 39401-55 | CM | Sorbose-sucrose | 191·0±27·3 | 0 | 930 |
| | | | 191·0±27·3 | 49·3±1·8 | 930 |
| K51 | CM | Sorbose-sucrose | 60·0±2·5 | 1·5 | 1100 |
| K9 | CM | Sorbose-sucrose | 60·0±2·5 | 15·0±2·1 | 370 |
| | | | 36·0±3·3 | 1·5 | 600 |
| K42 | CM | Sorbose-sucrose | 12·5±2·5 | 0 | 1500 |
| K26 | CM | Sorbose-sucrose | 11·0±1·9 | 3·8±1·1 | 29 |
| | | | 40·3±2·3 | 0 | 320 |
| K13·5 | CM | Sorbose-sucrose | 16·2±2·2 | 0 | 108 |

(Macroconidial strains: W40, 37401, K26-9 rows; Microconidial strains: 9a91, 39401-55, K51, K9, K42, K26, K13·5 rows.)

*Agar medium except where indicated.    †Size of colonies very much smaller than on control plates—see Plate 1, Figure 1.

Many of the experiments were performed on the low carbohydrate sorbose-sucrose medium but qualitatively similar results were obtained when 2 per cent. glucose minimal medium was substituted. The concentration of auxotrophs necessary to suppress prototrophs was much higher on the glucose than on the sorbose-sucrose medium. For example, $3 \times 10^6$ microconidia from a W40 adenineless culture were sufficient to suppress prototrophic microconidia completely when added to 10 ml of sorbose-sucrose medium, but $3 \times 10^7$ conidia were necessary when glucose medium was used.

It is apparent that macroconidia from auxotrophic cultures have a greater suppressing capacity than microconidia. 9a91 and W40 carry the adenine-purple gene, but 10 times the number of microconidia from 9a91 are necessary to suppress prototrophic conidia as macroconidia from W40. Also a higher number of auxotrophic conidia are needed to suppress macroconidia from the prototrophic strain 70007 than microconidia from CM.

It is of interest that a similar number of $ad$-purple microconidia from 9a91 were required to suppress prototrophic microconidia as $ad$-purple macroconidia from W40 to suppress prototrophic macroconidia 70007. A subinhibitory dose of W40 macroconidia did not reduce the number of prototrophic colonies produced but did cause a considerable reduction in their size compared with the controls (Table 2; Plate 1, Fig. 1).

Since the medium containing 2 per cent. glucose is the one most used in back-mutation experiments in *Neurospora* the results obtained with it deserve special attention. Complete suppression of prototrophic microconidia occurred when they were mixed with $3 \times 10^7$ W40 macroconidia and plated in 10 ml of glucose medium (Table 2). When reconstruction experiments were performed with macroconidia of W40 and of Kølmark and Westergaard's (1953) *circularis* strain, inhibition of the prototrophs, as shown by a decrease in colony diameter compared with the controls, was caused by $1.9 \times 10^7$ W40 conidia per plate. A five-fold decrease in colony size was induced by $3.7 \times 10^7$ W40 conidia. Many of the colonies were barely visible. Suppression of prototrophic macroconidia by conidia from W40 was observed in liquid medium containing 2 per cent. glucose. Thus despite Kølmark and Westergaard's (1952) claim to the contrary some inhibition of prototrophic cells by W40 conidia seems to occur at concentrations commonly used in mutation experiments.

Relatively low numbers of macroconidia ($1.7 \times 10^6$) per 10 ml of medium, from a histidineless strain (K26·9) caused a considerable inhibition of prototrophs on glucose medium. Both the number of colonies and their size were considerably diminished. A concentration of $2 \times 10^7$ K26·9 conidia was sufficient to produce complete suppression of macroconidial prototrophs when plated on a high carbohydrate conidiating medium containing 2 per cent. glycerol (Horowitz 1947).

These observations suggested that the apparent mutagenic activity of some treatments such as radiations might be explicable on the basis of their known lethal effects if the inhibition of prototrophs caused by the dead cells was smaller than that caused by living cells. Consequently several reconstruction experiments were performed in which dead W40 conidia, killed by ultraviolet irradiation, were mixed with prototrophic microconidia and plated on minimal medium. The numbers of

prototrophic colonies on the minimal and minimal plus dead conidia medium did not differ, nor were the prototrophic colonies reduced in size, even when as many as $5 \times 10^7$ dead conidia were added to each plate; on the contrary, the prototrophic colonies appeared much sooner after plating of conidia on the "minimal plus dead conidia" medium than on the minimal medium (Table 3). This acceleration of growth of prototrophs on medium containing dead conidia is of importance when considering the reliability of platings of untreated viable auxotrophic cells as controls of mutation experiments.

### (b) Dilution Experiments

Because wild-type conidia are inhibited by auxotrophic conidia, any prototrophic ones present as a result of spontaneous mutation in a population of auxotrophic cells should not grow on a plate containing minimal medium if an inhibiting concentration of auxotrophic conidia is present. If wild-type cells were in the mutant population then dilution of a cell suspension to a level less than the inhibitory one might allow wild-type colonies to appear.

TABLE 3

EFFECT OF DEAD W40 CONIDIA IN THE MEDIUM ON THE GERMINATION RATE OF PROTOTROPHIC CONIDIA

Number of prototrophic colonies per plate visible 3 days after the addition of equal numbers of prototrophic conidia to minimal medium and minimal medium plus dead cells

| Experiment | Minimal Medium | Minimal Medium plus Dead Cells |
|------------|----------------|-------------------------------|
| 1 | 0 | $6 \cdot 3 \pm 1 \cdot 4$ |
| 2 | $31 \cdot 8 \pm 6 \cdot 2$ | $62 \cdot 7 \pm 1 \cdot 5$ |
| 3 | $5 \cdot 8 \pm 0 \cdot 9$ | $14 \cdot 0 \pm 1 \cdot 5$ |
| 4 | 0 | $5 \cdot 5 \pm 1 \cdot 3$ |

From the results obtained dilution experiments varied considerably from strain to strain. In some, e.g. K42, the yields of prototrophs were greater at low concentrations than would be expected from the number which appeared at higher concentrations, in others, e.g. W40, a decrease in the number of conidia per plate resulted in a proportionate decrease in the number of prototrophic colonies detected (cf. Kølmark and Giles 1955). Variable results were obtained with other strains. Qualitatively similar data were observed whether the dilutions were performed on sorbose-sucrose or glucose medium. The data from one of the series of dilution experiments are shown in Table 4(a). The occurrence of prototrophs in this irradiated population of K42 cells can be adequately explained on the measured lethal effects of the radiation without assuming that the treatment was mutagenic (Table 4(b) and Plate 1, Fig. 2). Similar results were obtained when serial dilutions of conidia from some but not all 9a91 cultures were plated. The discrepancy may have arisen as a result of differences in the small proportion of macroconidia with their greater suppressing ability (10–50 times that of microconidia) in the otherwise microconidial population or simply because of a real difference in the proportion of prototrophs present in the populations plated.

Although no regular "dilution effect" was observed with macroconidia from strains W40 and 37401, occasionally larger numbers of prototrophs appeared in diluted samples than were expected from the numbers observed in the more concen-

TABLE 4

DILUTION AND IRRADIATION EXPERIMENTS

(a) Number of prototrophic colonies per plate visible 4 days after the plating of several concentrations of conidia from strains 9a91 (ad-purple) and K42 (leuc)

|  | Dilution | | | |
|---|---|---|---|---|
|  | 0 | 10 | $10^2$ | $10^3$ |
| **Strain K42** | | | | |
| Mean number of colonies per plate | 0·8 | 22·6±3·5 | c. 200 | 17·5±0·81 |
| No. of viable auxotrophic conidia per plate | $5 \times 10^7$ | $5 \times 10^6$ | $5 \times 10^5$ | $5 \times 10^4$ |
| **Strain 9a91** | | | | |
| (i) Mean number of colonies per plate | 0 | 106·3±21·4 | 3·2±1·9 | — |
| No. of viable auxotrophic conidia per plate | $5 \times 10^7$ | $5 \times 10^6$ | $5 \times 10^5$ | — |
| (ii) Mean number of colonies per plate | c. 1000* | 249·0±49·0 | 22·0±4·0 | 1·0 |
| No. of viable auxotrophic conidia per plate | $2·1 \times 10^7$ | $2·1 \times 10^6$ | $2·1 \times 10^5$ | $2·1 \times 10^4$ |

(b) Number of prototrophic colonies appearing after ultraviolet irradiation of K42 conidia compared with those appearing when unirradiated conidial suspensions are diluted and plated

|  | Unirradiated | | | | Ultraviolet Irradiated | |
|---|---|---|---|---|---|---|
|  | Dilution | | | | Dilution | |
|  | 0 | 10 | $10^2$ | $10^3$ | 0 | 10 |
| Mean number of colonies per plate | 0 | 45·6±2·4 | c. 500 | 42·2±7·9 | Large number | 91·5±2·5 |
| No. of viable auxotrophic conidia per plate | $2·0 \times 10^7$ | $2·0 \times 10^6$ | $2·0 \times 10^5$ | $2·0 \times 10^4$ | $11·0 \times 10^5$ | $11·0 \times 10^4$ |
| No. of colonies per $10^6$ auxotrophs plated | 0 | 22·8 | c. 2500 | 21·10 | — | 827 |

*Minute colonies, appeared several days after others.

trated samples. In multinucleate macroconidia there is the possibility that auxotrophic nuclei interfere with the expression of prototrophic ones sharing the same cytoplasm (Grigg 1952). Some evidence of this interference is given below.

TABLE 5

INTRACONIDIAL INTERACTION BETWEEN PROTOTROPHIC AND AUXOTROPHIC COLONIES

Number of colonies per petri plate after incubation of conidia from two histidineless prototrophic heterokaryons at 25°C

(a) Heterokaryon 1*     $h^+ : h :: 1 : 2$

| | Sorbose Minimal Medium | | | | Sorbose Minimal Medium plus 30 μg/ml Histidine | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Days of Incubation | | | | Days of Incubation | | | |
| | 3 | 4 | 5 | 6 | 3 | 4 | 5 | 6 |
| Total | 64 | 302 | 383 | 415 | 278 | 463 | 527 | 545 |
| Mean | 16.0±3.7 | 75.5±6.2 | 95.7±5.9 | 103.7±5.5 | 69.5±4.3 | 115.7±6.0 | 131.7±6.3 | 136.2±6.6 |
| Mean of estimated number of prototrophic colonies per plate | 16.0±3.7 | | | 103.7±5.5 | 50.5±2.8 | | | 99.5±4.3 |

Mean number of "late" colonies    $103.7\pm5.5 - 16.0\pm3.7 = 87.7\pm8.5$      $99.5\pm4.3 - 50.5\pm2.8 = 49.0\pm1.8$

(b) Heterokaryon 2†     $h^+ : h :: 2 : 1$

| | Sorbose Minimal Medium | | | | Sorbose Minimal Medium plus 30 μg/ml Histidine | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Days of Incubation | | | | Days of Incubation | | | |
| | 3 | 4 | 5 | 6 | 3 | 4 | 5 | 6 |
| Total | 59 | 223 | 283 | 299 | 51 | 131 | 177 | 211 |
| Mean | 14.7±2.3 | 55.7±5.5 | 70.7±2.0 | 74.7±2.5 | 17.0±6.7 | 43.7±3.9 | 59.0±3.0 | 70.3±1.2 |
| Mean of estimated number of prototrophic colonies per plate | 14.7±2.3 | | | 74.7±2.5 | 17.0±6.7 | | | 67.3±1.2 |

Mean number of "late" colonies    $74.7\pm2.5 - 14.7\pm2.3 = 60.0\pm3.7$      $67.3\pm1.2 - 17.0\pm6.7 = 50.3\pm6.8$

*Viability of conidia plated = 80 per cent.      †Viability of conidia plated = 60 per cent.

### (c) *Intracellular Suppression*

The second assumption of the back-mutation assay procedure, that there is no intracellular suppression in multinucleate cells or phenotypic lag in uninucleate cells, is more difficult to test than the first. Some information can be obtained by studying germination of conidia from heterokaryons of the constitution $a–a^+$ where $a$ is an auxotrophic mutant. Such studies with artificial heterokaryons have indicated that under certain conditions auxotrophic nuclei interfere with the free expression of the wild-type nuclei.

The metric used was the rate of appearance of macroscopic colonies. This rate was expressed as the number of heterokaryotic colonies visible macroscopically on minimal and on supplemented medium at times $t_1$, $t_2$, etc. If the auxotrophic nuclei in the heterokaryotic conidia did not affect the expression of the wild-type nuclei the germination on minimal and supplemented medium should be equal to that of homokaryotic wild-type conidia on the same media.

Aliquots of conidia from two *hist–hist*$^+$ heterokaryons differing only in their nuclear ratios were plated on minimal medium and on histidine-supplemented medium. The colonies were labelled as they appeared and the numbers recorded during the following 6 days. The data from the plating experiments are recorded in Table 5.

An estimate of the number of prototrophic colonies (both hetero- and homo-karyotic) on the histidine-supplemented plates can be obtained by subtracting from the total on each day the calculated proportion of histidineless colonies, assuming equal germination rates of wild-type and *hist* histidineless conidia on histidine-supplemented medium when tested. Actually the *hist*$^+$ conidia germinated more rapidly than the *hist* ones so this simplification will tend to decrease in magnitude any observed differences between them. The estimated numbers, $n_1'$ and $n_2'$ of proto-trophic (hetero- and homokaryotic) colonies per plate after incubation for 3 days are given in Table 5.

In heterokaryons 1 and 2 grown on complete medium, the proportion of hetero-karyotic conidia expected on the assumption of random segregation of nuclei into the multinucleate conidia is 68 per cent. (estimated by the method of Prout *et al.* 1953). Seven per cent. in heterokaryon 1 and 25 per cent. in heterokaryon 2 were expected to be wild type. Hence even though it was not possible to distinguish wild type from heterokaryotic colonies in the data the fact that the latter were far more frequent makes it possible to detect a lowered germination rate of the heterokaryons on minimal medium should it exist.

It is apparent from the data in Table 5 that in heterokaryon 1 the number of prototrophic colonies visible on the third day of incubation was much lower on minimal than on histidine-supplemented medium although the final numbers of hetero-karyotic colonies visible on the sixth day on both media were equal. The difference is highly significant ($P \leqslant 0.01$). On the other hand, no difference was apparent with the second heterokaryon. It will be recalled that this heterokaryon had a higher proportion of wild-type to histidineless nuclei than the other one. This second plating experiment demonstrated that the addition of histidine to the medium did not affect

the germination rate of wild-type conidia or conidia from a heterokaryon having only a low proportion of histidineless nuclei.

These experiments are interpreted to show that if present in the proportion $hist^+ : hist :: 1 : 2$, histidineless nuclei interfere with the free expression of the $hist^+$ nuclei, whereas no such effect is demonstrable when $hist^+ : hist :: 2 : 1$.

Similar experiments were performed with an arginineless–tyrosineless heterokaryon, the components of which carried complementary albino markers and additional biochemical mutant genes. The heterokaryon had the wild-type colour (pink). No difference in germination rate was observed between the minimal medium and media supplemented with arginine, tyrosine, or arginine and tyrosine.

TABLE 6

MECHANISM OF SUPPRESSION 1

Colony counts after incubation of plates at 25°C for 5 days, showing the failure of growth of prototrophic conidia on a culture filtrate of K26·9 or W40 conidia in minimal medium, compared with that on a medium consisting of the filtrate plus sugars

|  | Agar, K26·9 Filtrate* | Agar, Sugars, K26·9 Filtrate* | Agar, W40 Filtrate† | Agar, Sugars, W40 Filtrate† |
|---|---|---|---|---|
| Total | 81‡ | 139 | 5‡ | 156 |
| Mean | 20·2 + 12·4 | 34·8 ± 3·9 | 1·7 ± 1·2 | 39·0 ± 4·4 |

*The culture filtrate from a suspension of $2·5 \pm 0·3 \times 10^7$ K26·9 conidia was added to each petri plate of medium.

†Each petri plate of medium contained the culture filtrate from a suspension of $3·95 \times 10^7$ W40 conidia.

‡Size of colonies much less than in other group.

### (d) Mechanism of High Density Suppression

Two general mechanisms by which one cell type may suppress another are (1) the inhibiting species may produce some substance toxic to the other cell species, (2) it may remove some substance from the medium which the other cell type requires for growth. That the culture filtrate from medium in which an inhibiting dose of K26·9 conidia had been incubated for 4 days contained no appreciable content of toxic substance was apparent when its addition to fresh medium produced no obvious inhibition of colony growth.

On the other hand, addition of fresh salts and glucose to a culture filtrate which did not support growth of a prototrophic inoculum resulted in immediate resumption of normal growth. Washed conidia were incubated in 8-ml aliquots of liquid minimal medium for 3 days at 25°C. The filtrate from each culture was heated to 95°C for 15 min prior to mixing with agar (0·2 g) alone or plus sugar. After cooling to 40°C aliquots of prototrophic conidia were added, the mixture poured into petri dishes, and incubated at 25°C. Sugar added alone to culture filtrates of a histidineless K26·9 and an adenineless W40 strain was sufficient to produce normal growth of prototrophic conidia inoculated, as is shown in Table 6.

It has been observed (Table 2) that a concentration of auxotrophic conidia sufficient to suppress prototrophic conidia completely on a low-sugar medium caused no observable suppression on a high-sugar (2 per cent.) medium. Therefore auxotrophic conidia (K26·9) were incubated in media containing a low (0·2 per cent.) and a high (2·0 per cent.) concentration of sugar. Prototrophs added subsequently did not grow to macroscopic size on the low-sugar culture filtrate, but growth was quite normal on the high-sugar culture filtrate medium as it was on low-sugar filtrate plus added sugar medium (Table 7). Presumably the auxotrophs removed most of the sugar from the low-sugar medium, but only a proportion of that in the high-sugar medium leaving sufficient for normal growth of the prototrophs.

### TABLE 7
#### MECHANISM OF SUPPRESSION 2

Growth of prototrophic conidia on a culture filtrate from a suspension of K26·9 conidia in a low-sugar minimal medium (0·2 per cent.) compared with that on a high-sugar minimal medium (2·0 per cent.), sugar-supplemented culture filtrate, and minimal medium. Equal aliquots of prototrophic conidia were added to all plates of medium. Colony counts after incubation of plates at 25°C for 5 days

|  | Minimal Medium | Agar, K26·9 Filtrate (low sugar)* | Sorbose-sucrose, K26·9 Filtrate (low sugar)* | Agar, K26·9 Filtrate (high sugar)* | Sorbose-sucrose, K26·9 Filtrate (high sugar)* |
|---|---|---|---|---|---|
| Total | 111 | 1 | 194 | 56 | 65 |
| Mean | 27·8±3·7 | 0·25±0·25 | 48·5±7·6 | 18·7±0·9 | 21·7±1·5 |

*Each petri plate of medium contained the culture filtrate from a suspension of $2·8 \times 10^7$ K26·9 conidia.

A periodical sampling of a suspension of auxotrophic conidia in liquid minimal medium and a manometric assay of the glucose content of the sample using glucose oxidase verified this presumption. Samples were removed simultaneously from flasks containing only minimal medium and minimal plus auxotrophic conidia. In the absence of inositol, conidia from the inositolless strain 37401 removed $2·52 \times 10^{-7}$ mg glucose per day from the medium in a linear fashion for 4 days when uptake ceased abruptly. The uptake from inositol-supplemented medium followed the same curve as the unsupplemented one for the first 2 days, but thereafter increased rapidly.

## IV. DISCUSSION

### (a) The Suppression Phenomenon

Of the factors contributing to suppression of prototrophs by auxotrophs perhaps the most important are (i) the removal of sugar by the auxotroph, and (ii) the growth rate of the prototroph.

(i) *Sugar Uptake by Auxotrophs.*—Since the addition of sugar to a culture filtrate from a suspension of non-growing auxotrophic conidia in minimal medium restored the ability of the filtrate to support the growth of prototrophic conidia, plus the fact

that the auxotrophs took up sugar from the medium, it seems likely that removal of sugar from the medium by the auxotrophic cells was involved in the mechanism of the suppression. The sudden drop in sugar uptake of the inositol-requiring strain 37401 after 3 days on minimal medium was probably due to death of most of the conidia. It is of interest that in the first 2 days after inoculation the inositolless conidia removed from the medium the same amount of sugar on minimal as they did on inositol-supplemented medium.

If the sugar level in the minimal medium is reduced below that necessary for growth of the prototrophs before they have reached macroscopic colony size no prototrophs will be detected macroscopically. An increase in the sugar content of the plating medium diminishes the suppresssion effect as does a decrease in the number of inhibiting auxotrophic cells present.

Ryan and Schneider (1949) using *Esch. coli* have shown that under anaerobic conditions the suppression of prototrophs by histidine-requiring bacteria was brought about by a somewhat similar mechanism.

(ii) *Growth Rate of the Prototroph.*—It might be anticipated that the germination and growth rates of the particular prototrophic species present would be an additional important factor. The observation that a greater concentration of auxotrophic conidia must be present to suppress the prototrophic macroconidia than the more slowly germinating microconidia confirms this.

Prototrophs grew more rapidly on medium containing radiation-killed cells than on medium containing none (Table 3). It has been demonstrated that even non-lethal irradiation can stimulate growth (Adelstein *et al.* 1952). Thus prototrophs present in an irradiated cell population might be expected to form macroscopic colonies sooner than prototrophs in a non-irradiated population and hence have a lesser chance of being suppressed by a given number of auxotrophs than the latter.

In back-mutation experiments with macroconidial strains of *Neurospora*, prototrophs are usually in the form of heterokaryons with nuclei of the parental auxotroph. In estimating the importance of intercellular suppression in such a situation by means of reconstruction experiments this fact should be recognized since, as we have seen with histidineless wild-type heterokaryons, heterokaryotic conidia may grow or germinate more slowly than prototrophic homokaryons on minimal medium. The extent of the intracellular inhibition increased with increase in proportion of histidineless nuclei, within the restricted range of nuclear ratios used. It is not unreasonable to suppose that heterokaryotic conidia from a histidineless strain containing a very low proportion of spontaneous back-mutants would show an even slower growth on minimal medium. Growth might be so slow that such conidia would not form macroscopic colonies before the experiment were concluded and hence would not be scored. Irradiated heterokaryons will have, on the average, a lower number of inhibiting auxotrophic nuclei per conidium and consequently might be expected to have a more rapid germination rate. Again, the small amounts of growth substance released into the medium from killed cells would tend to increase the germination rate of the treated heterokaryotic conidia compared to the non-treated ones.

These various effects all tend to increase the chance of prototrophic nuclei, present before treatment, forming macroscopic colonies on treated plates as compared

with the control plates. Account should be taken of them when performing control reconstruction experiments to test for competitive suppression.

### (b) *Mutation Assay in* Neurospora

It has been claimed that W40 *circularis* conidia do not inhibit prototrophs on 2 per cent. glucose medium (Kølmark and Westergaard 1952). We found, however, that the maximum concentration of W40 conidia used by these workers caused inhibition of growth of added prototrophic conidia on this medium (shown as a considerable reduction in size of prototrophic colonies compared with the controls). No prototrophic colonies appeared when double this concentration of W40 conidia was used. The suppression of prototrophic conidia by auxotrophic ones at concentrations commonly used in mutation experiments has been confirmed by Stephens and Mylroie (1953).

TABLE 8
BACK-MUTATION RATE OF INOSITOLLESS CONIDIA FOLLOWING
IRRADIATION WITH ULTRAVIOLET AND VISIBLE LIGHT
Data extracted from Table 1 of Brown (1951)

| Dosage of Ultraviolet (ergs/mm²) | Number of Survivors Tested ($\times 10^{-6}$) | Reversion Frequency per $10^6$ Viable Cells |
|---|---|---|
| 1500 | 26 | 9 |
| | 50 | 4·5 |
| | 52 | 4 |
| 2000 | 13 | 14 |
| | 25 | 8 |
| | 26 | 3·7 |
| 2500–3000 | 1 | 135 |
| | 2·5 | 96 |
| | 10 | 25 |
| | 13 | 8·3 |

Anomalous results in microbial mutation experiments reported in the literature can be explained readily by suppression of rare wild-type cells by the mutant ones. Thus the observation of Brown (1951) that as many prototrophic colonies appeared at 1/100 as at 1/10 dilution when microconidia from an inositol-requiring strain were plated on minimal medium is strongly suggestive of high density suppression. A similar dilution phenomenon was described for some other microconidial auxotrophs by Grigg (1952) and also in this paper. In these the growth of prototrophic conidia derived by spontaneous mutation was suppressed on the plates containing the greatest density of auxotrophs, but as the density of auxotrophs decreased the number of visible prototrophic colonies increased. A comparison of the mutation rates given in Table 1 of Brown (1951) resulting from each treatment with the number of cells plated suggests a relation between the number of survivors tested and the reversion frequency (Table 8). The reversion frequency seems independent of the dosage and dependent

only on the numbers of survivors. When $26 \times 10^6$ or more survivors were plated the reversion frequency was moderately low but when fewer than $13 \times 10^6$ survivors were plated the "reversion frequency" increased very considerably. Because of the likelihood that competitive suppression was influencing her results Brown's interpretation of them may be queried.

It is apparent that cell interactions of the type discussed here can seriously bias interpretation of data in mutation experiments, but do they invalidate the claims that various chemicals and irradiations increase the back-mutation rate in microorganisms? No general answer can be given to this since most workers have ignored the necessity to test the assumptions inherent in their techniques of recovering "induced" reversions. Of the published work in which an attempt has been made to do this, that of Giles (1951) and Kølmark and Giles (1955) is the most impressive and Giles' interpretation, that various treatments including ionizing radiations increased the back-mutation frequency, therefore, seems reasonable.

Irradiation does not increase the back-mutation rate of all biochemical mutants of *Neurospora* as was illustrated by the observations on the nicoticless strain 39401; in fact, those on which most of the published work has been done, namely 37401 and W40, are probably selected samples. It may well be that a search will locate numerous loci in *Drosophila* and maize which show a behaviour to irradiation similar to *Neurospora* genes 37401 and W40. The differences in the genetic behaviour to irradiation between microorganisms and higher organisms which have been claimed (Lefevre 1950) may prove fallacious.

### (c) *Bacterial Plating Experiments*

Competitive suppression seems of widespread occurrence in experiments with bacteria. During the course of an elegant series of experiments on mutation in a histidine-dependent strain of *Esch. coli*, Ryan and Schneider (1949) found that prototrophs did not multiply in the presence of $7 \times 10^9$ histidine-dependent cells per 5 mg glucose in the medium. The authors commented that the growth restriction observed may be of general occurrence in mixtures of auxotrophic and prototrophic cells. That a similar phenomenon operates in mixtures of cells sensitive and resistant to drugs and phage is apparent. For instance Saz and Eagle (1953) observed that the addition of penicillin-sensitive bacteria prevented the growth of penicillin-resistant ones in the presence of penicillin. In explanation they postulated a mechanism involving the release of a species-specific toxic factor from the inactivated sensitive cells which killed the resistant ones. It seems more likely that the inactivated cells inhibited growth of the resistant ones, perhaps by removing a nutrient from the medium. Newcombe (1948) reported an apparent decline in the mutation rate of phage-sensitive to phage-resistant *Esch. coli* cells when densities greater than $5 \times 10^9$ sensitive cells per plate were used. An increase in the number of back-mutant colonies with increase in concentration of mutant cells plated has been observed in several different microorganisms, e.g. *Esch. coli* (Bryson 1948), *Pseudomonas fluorescens* (Engelsberg 1952), *Brucella abortus* (Olitzki 1952, 1953). Despite the varied explanations put forward by these authors to explain their observations the simplest is that back-mutant cells present in the initial cell population were suppressed when the

concentration of cells plated exceeded a certain threshold. Since this threshold is likely to vary with the medium used it is not valid to carry out controls of the plating technique on one medium and to extrapolate the results to another.

Jinks (1952) has suggested that competitive suppression similar to that described in this paper could bias *Esch. coli* linkage data. Some data of Nelson (1951) in which an apparent increase in prototroph recombinants was associated with a decrease in the concentration of plated auxotrophs could be interpreted to support this view. In spite of these possible misinterpretations, it is unlikely that competitive suppression has been an important source of error in the interpretation of recombination experiments (Ryan 1953).

Competitive suppression seems of widespread occurrence in microorganisms. In consequence, adequate control reconstruction experiments should be part of any experiments where the frequency of one rare cell type is to be determined by plating methods.

## V. Acknowledgments

## VI. References

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## Explanation of Plate 1

Fig. 1.—Effect of $ad-3$ (W40) conidia on the growth of $ad-3^+$ conidia on minimal medium. All three plates contain an equal number of $ad-3^+$ conidia and the following numbers of $ad-3$ conidia: (a) none; (b) $2 \times 10^7$; (c) $4 \times 10^7$.

Fig. 2.—The number of *leuc* $^+$ colonies which appear on minimal medium when various numbers of *leuc* (K42) conidia are added to petri plates is compared with the number which arise following the plating of irradiated conidia on a similar medium. The number of *leuc* conidia per plate is: (a) $5 \times 10^7$, including the 95 per cent. killed by the irradiation; (b) $5 \times 10^7$; (c) $5 \times 10^6$; (d) $5 \times 10^5$; (e) $5 \times 10^4$; (f) $5 \times 10^3$.

DETECTION OF MUTATIONS IN MICROBIAL POPULATIONS
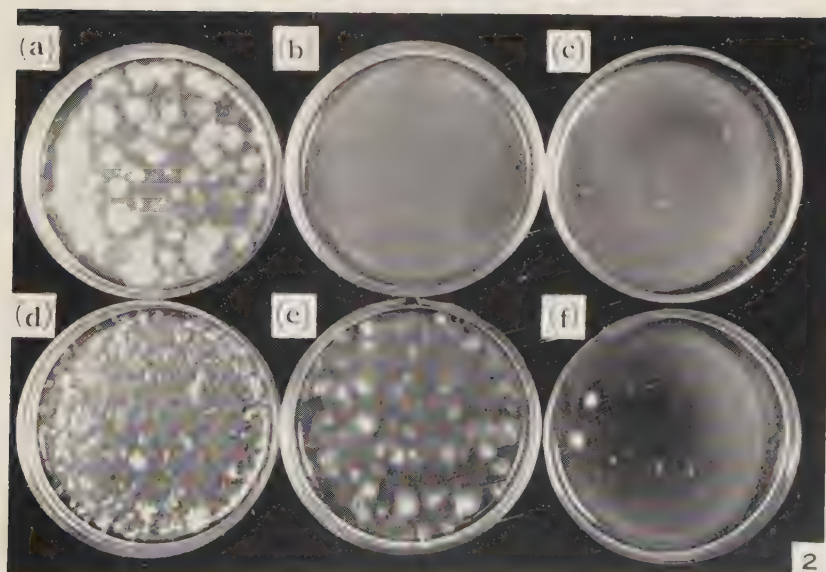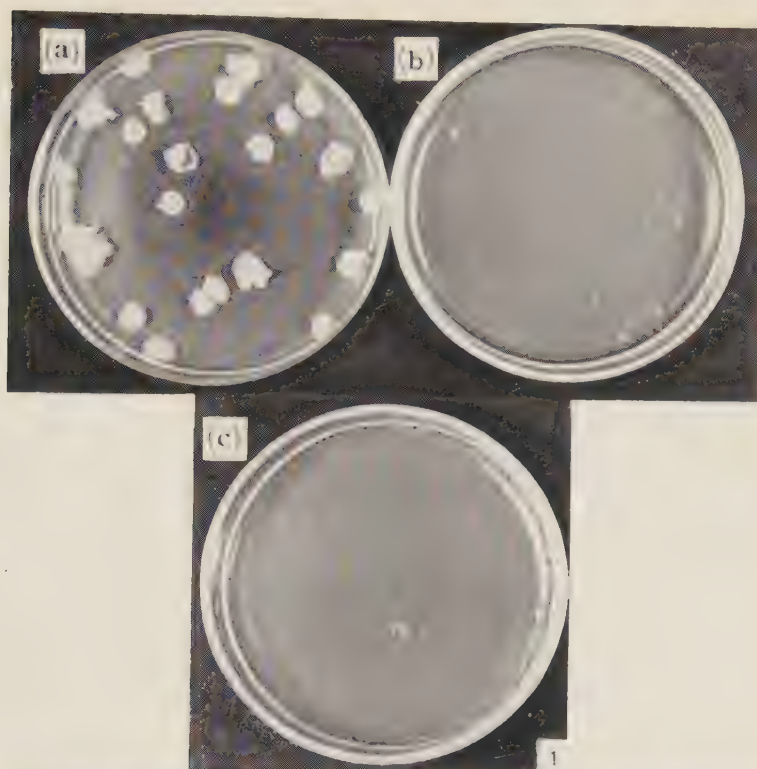
# THE EFFECT OF TEMPERATURE ON THE MUTATION RATE IN *DROSOPHILA MELANOGASTER*

By B. L. Sheldon*

## Summary

The incidence of sex-linked recessive lethal mutations in *Drosophila melanogaster* after heat shock treatment of both larvae and adult males is reported. There was no increase in the mutation rate after treatment of larvae and the results with adult males were not consistent. Treatment of the latter at 38°C caused an increase in mutation rate, due apparently to the large response of a few sensitive males. Treatment at 40°C caused no increase, and if one sensitive male was excluded, the mutation rate was significantly less than control. These results do not entirely support those of previous workers in the literature and possible reasons for this are discussed.

The mutation rate has also been studied, over a series of successive daily mating periods, of males undergoing development at three different temperatures. There was a significant regression (both linear and quadratic) of mutation rate on age, mutation rate decreasing with age of male, and this age effect did not differ between temperatures.

The linear regression of mutation rate on temperature was significant, mutation rate *decreasing* with increased temperature. Previous results in the literature have supported the opposite conclusion that mutation rate *increases* with increased temperature. It was postulated that the previous results may have been due to confounding with the effect of temperature on storage of mature sperm. The present results indicate that temperature during development has no direct effect on the mutation rate, since the higher rates with lower temperature are probably a function of longer developmental time at the lower temperature.

## I. Introduction

Plough (1941) reviewed the early literature on the influence of temperature on the mutation rate in *Drosophila melanogaster*, the first demonstration of an effect having been given by Muller with the introduction of his methods for the detection of lethal mutations.

The results of Muller (1928), Timofeeff-Ressovsky (1935), and Plough (1939) (the latter as reported in Plough (1941)), all showed that the mutation rate increases with increased temperature during development. Further results of Buchmann and Timofeeff-Ressovsky (1935, 1936) and Plough, Child, and Ives (Plough 1941) showed increased mutation rates after high temperature shocks for short periods both in larval and adult stages. Birkina (1938) and Kerkis (1941) obtained increases in the mutation rate after extremely low temperature shocks. The latter finding was supported by the results of Byers (1954), but Rendel and Sheldon (1956), in attempting to repeat the work of Birkina and Kerkis, obtained completely negative results.

In view of the many results showing an affect of age of adult and storage of sperm (Muller 1946; Lamy 1947; Mossige 1955), and temperature during sperm storage (Byers and Muller 1952; Byers 1954) on mutation rate, it is apparent that

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in many of the earlier experiments the effects due to temperature could have been confounded with the effects of some or all of these other processes. Rendel and Sheldon (1956) made allowances for this difficulty to some extent by ensuring that as far as possible control and treated flies were of about the same age group when mated to a tester stock. The same procedure has been followed in the heat shock experiments reported here, while the design of the present experiment on temperature during development attempts to remove completely any confounding with age of adult or storage of sperm.

Previous results showing an increase in mutation rate with increased temperature during development have generally been explained in terms of increased rates of chemical reactions at the higher temperatures. Results with temperature shocks (high and low) were not interpreted in the same way and a completely different set of phenomena was thought to be involved. The present experiments were planned firstly to repeat and extend the heat shock work of Buchmann and Timofeeff-Ressovsky, and secondly to study the effect of temperature during development, with the confounding effects of age of adult, storage of sperm, and different temperatures during such storage removed. From this detailed study of temperature during development, stages in the life cycle more mutagenically sensitive to such treatments and those most susceptible to temperature shocks may be found.

## II. Materials and Methods

The flies used in these experiments came from the same Oregon R-C wild-type stock of *D. melanogaster* as was used by Rendel and Sheldon (1956), and the same control results of spontaneous mutation rate at 25°C have been used here for comparison with results under the different heat shock treatments. Mutation rate in all cases was scored as the percentage of sex-linked recessive lethals, obtained by the standard procedure of mating males to be tested to females of a tester stock (Muller–5). An $F_2$ culture was scored as a lethal if no red-eyed males occurred out of 30 or more adults. In doubtful cases the test was carried on to an $F_3$. All cultures were kept at 25°C except during actual treatments.

### (a) Heat Shock Treatments

Heat shock was applied to both larvae and adult males. In the former case adult females were allowed to lay eggs in ordinary culture bottles for a 1-hr period during the afternoon and then removed. Cultures were inspected the following morning when many larvae were present. Heat shock treatment was applied to these cultures exactly 3 days later, so that treated larvae were in their fourth day of life. The cultures were treated in a water-bath at 36·5–38°C for periods of 1, 6, 12, and 24 hr (Buchmann and Timofeeff-Ressovsky (1935, 1936) had treated 3–5-day-old larvae at 35–38°C for 12–24 hr). Of the adults arising from the treated cultures only the males were tested for mutation rate.

For the heat shock treatment of adult males, the flies were placed either in sealed ampoules or empty stoppered 4 by 1-in. specimen tubes in water-baths at 38°C for 15 min, or at 40°C for 15–30 min (Buchmann and Timofeeff-Ressovsky had treated males and females at 36–39°C for 12–24 hr).

Except where otherwise indicated males were a random group up to 4 days old when mated to tester females in a single test-mating for each male. Usually one but

sometimes two tester females were allowed to each male. When adult males were treated they were mated immediately after treatment.

### (b) Temperature during Development

Adult females were allowed to lay eggs in a number of culture bottles for a 1-hr period at 25°C, after which they were removed, and several cultures were then placed in constant temperature rooms at each of three different temperatures—20, 25, and 30°C. The first progeny emerged at 13–14, 9–10, and 7 days respectively. The first 30, 24, and 20 males emerging were immediately set up individually with about eight Muller–5 tester females. At 24-hr intervals the males were transferred to fresh batches of tester females, the number of females used varying from eight in the first few days down to five or six at about the fifteenth day. This type of mating

TABLE 1
MUTATION RATES AFTER HEAT SHOCK OF LARVAE

| Treatment on Fourth Day | | No. of X-chromosomes Tested | No. of Recessive Lethals | Lethals (%) | Fiducial Limits (5% level) |
|---|---|---|---|---|---|
| Temperature Range (°C) | Time (hr) | | | | |
| Control | | 6410 | 15 | 0·23 | 0·13–0·39 |
| 36·5–38 | 1 | 1489 | 7 | 0·47 | 0·19–0·97 |
| 36·5–38 | 6 | 1387 | 3 | 0·22 | 0·05–0·63 |
| 36·5–38 | 12 | 1425 | 4 | 0·28 | 0·08–0·72 |
| 36·5–38 | 24 | None survived | — | — | — |

procedure was followed to ensure as far as possible that each day a newly matured sample of sperm was being tested (Mossige 1955), and so avoid confounding of the main age and temperature effects by the effects of storage of sperm at different temperatures.

All test matings were carried out at the temperature at which the males developed. In other words, males developing at 30°C would spend the rest of the period of the experiment at that temperature except for the short time each day involved in transferring to fresh batches of females. The latter was all done at room temperature which on most occasions was not greater than 25°C. After removal of the males the testing procedure beginning with fertilized tester females was carried through to the $F_2$ at 25°C.

The three different temperature treatments were done at different times, because the available laboratory facilities would not allow them to be handled concurrently. For similar reasons in the 25 and 30°C treatments males could be tested only till the fifteenth day of age, whereas in the 20°C treatment they could be tested till the twenty-second day.

### III. RESULTS

### (a) Heat Shock

The results obtained after heat shock in the larval stage are given in Table 1. Whereas Buchmann and Timofeeff-Ressovsky indicated that they had obtained some results after a shock period of 24 hr, this could not be repeated in the present

experiment, as larvae so treated all died.   No attempt was made to get mortality rates for larvae in the other treatment periods, but it was observed that adults were over a day later emerging in the 12-hr treated cultures than in the 1- or 6-hr treatments. In addition, less adults were obtained from the cultures treated for 12 hr.   The main feature of the results is that none of the treatments differs significantly from the control.   There is an indication of an increase (not significant, $\chi^2_1 = 1\cdot6$) for the larvae treated for 1 hr and this will be considered further in Section IV.

Table 2 shows the results obtained after heat shock of adult males.   The treatment at 40°C was carried out because it was close to the most extreme shock possible without killing all flies.   Mortality under this treatment varied between 20 and 50 per cent., and fertility of survivors was greatly reduced, up to 70 per cent. being completely sterile.   After treatment at 38°C for 15 min all males survived and their fertility was not obviously affected.

TABLE 2

MUTATION RATES AFTER HEAT SHOCK OF ADULT MALES

| Treatment | No. of X-chromosomes Tested | No. of Recessive Lethals | Lethals (%) | Fiducial Limits (5% level) |
|---|---|---|---|---|
| Control | 6410 | 15 | 0·23 | 0·13–0·39 |
| 38°C for 15 min | | | | |
| Replicate 1 | 2468 | 28 | 1·13 | 0·76–1·64 |
| Replicate 2 | 1533 | 7 | 0·46 | 0·18–0·94 |
| 40°C for 15–30 min | | | | |
| Replicate 1 | 918 | 1 | 0·11 | 0·003–0·61 |
| Replicate 2 | 1809 | 0 | 0·00 | 0·00–0·20 |
| Replicate 3 | 1922 | 8 | 0·42 | 0·18–0·82 |

There is a significant difference between the two replicates of the 38°C treatment ($\chi^2_1 = 6\cdot1$), the very high value for the first replicate being due to the extremely high mutation rate of two of the males out of the 40 used in this replicate.   Thus one of these males had nine recessive lethals out of 35 tested X-chromosomes, and the other had 12 lethals out of 77 tested X-chromosomes.   This marked clumping of lethals did not occur in the second replicate, where only one male out of 55 treated had more than 3 per cent. lethals and then only four lethals out of 40 tested X-chromosomes.   Replicate 1 is significantly higher than the controls ($\chi^2_1 = 16\cdot5$, $P<0\cdot001$), but there is no significant difference between replicate 2 and controls ($\chi^2_1 = 1\cdot5$).

If the results of the 40°C replicates are pooled, the result (0·19 per cent. lethals) is not significantly different from control ($\chi^2_1 = 0\cdot205$).   There is, however, a significant difference among the three replicates of this treatment ($\chi^2_2 = 8\cdot94$, $P<0\cdot02$).   Replicates 1 and 3 are not significantly different from control, but replicate 2 is significantly lower than the control at the 0·1 per cent. level ($\chi^2_1 = 14\cdot7$).

### (b) Temperature during Development

The results for the three treatments over all ages tested are given in Table 3. The values for the number of X-chromosomes tested give some indication of the

TABLE 3

EFFECT OF AGE OF MALE AND TEMPERATURE DURING DEVELOPMENT ON THE MUTATION RATE

| Age of Males (days) | 20°C | | | 25°C | | | 30°C | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. of X-chromosomes Tested | No. of Recessive Lethals | Lethals (%) | No. of X-chromosomes Tested | No. of Recessive Lethals | Lethals (%) | No. of X-chromosomes Tested | No. of Recessive Lethals | Lethals (%) |
| 1 | 126 | 1 | 0·79 | 615 | 3 | 0·49 | 310 | 4 | 1·29 |
| 2 | 482 | 4 | 0·83 | 1010 | 6 | 0·59 | 900 | 2 | 0·22 |
| 3 | 773 | 5 | 0·65 | 1036 | 3 | 0·29 | 797 | — | — |
| 4 | 901 | 1 | 0·11 | 939 | 4 | 0·43 | 826 | 1 | 0·12 |
| 5 | 1007 | 1 | 0·10 | 849 | 3 | 0·35 | 646 | — | — |
| 6 | 1082 | 3 | 0·28 | 1000 | 1 | 0·10 | 638 | 2 | 0·31 |
| 7 | 535 | — | — | 929 | 2 | 0·22 | 453 | — | — |
| 8 | 435 | 1 | 0·15 | 821 | 1 | 0·12 | 214 | — | — |
| 9 | 670 | — | — | 756 | — | — | 172 | — | — |
| 10 | 672 | — | — | 744 | 1 | 0·13 | 147 | — | — |
| 11 | 533 | 2 | 0·38 | 617 | — | — | 32 | — | — |
| 12 | 499 | 1 | 0·20 | 613 | — | — | 46 | — | — |
| 13 | 555 | — | — | 552 | 1 | 0·18 | 21 | — | — |
| 14 | 440 | 1 | 0·23 | 545 | — | — | 13 | — | — |
| 15 | 278 | — | — | 446 | — | — | | | |
| 16 | 292 | — | — | | | | | | |
| 17 | 282 | 1 | 0·35 | | | | | | |
| 18 | 294 | — | — | | | | | | |
| 19 | 294 | — | — | | | | | | |
| 20 | 251 | — | — | | | | | | |
| 21 | 194 | — | — | | | | | | |
| 22 | 269 | — | — | | | | | | |
| To 15th day | 8988 | 20 | 0·223 | 11,472 | 25 | 0·218 | 5215 | 9 | 0·173 |

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decline in fertility with age in all three treatments, since the experiment was planned
to test approximately 1000 chromosomes per day per treatment, granted normal
fertility, from 20–30 males per treatment.   This drop in number of chromosomes
tested was, however, not only a reflection of lower fertility with age under these
conditions of mating, but was also due in part to a certain proportion of flies dying
or being lost during transfers over the course of the experiment.   The much lower
number of tested chromosomes in the 30°C treatment was due to an extreme degree
of infertility and mortality in the adult stage at this temperature.

TABLE 4

ANALYSIS OF VARIANCE OF SQUARE ROOT TRANSFORMATIONS OF PERCENT-
AGE LETHAL FREQUENCIES IN TABLE 3

| Source of Variation | D.F. | Mean Square | $F$ |
|---|---|---|---|
| Between temperatures | (2) | | |
|   Linear | 1 | 0·3435 | 5·90* |
|   Quadratic | 1 | 0·0495 | 0·85 |
| Between ages | (14) | | |
|   Linear | 1 | 1·9421 | 33·37*** |
|   Quadratic | 1 | 0·4230 | 7·27* |
|   Remainder | 12 | 0·0282 | |
| Interaction | | | |
|   Linear × linear | 1 | 0·0024 | 0·04 |
|   Linear × quadratic | 1 | 0·0012 | 0·02 |
|   Quadratic × linear | 1 | 0·0114 | 0·20 |
|   Quadratic × quadratic | 1 | 0·0900 | 1·55 |
| Error | 24 | 0·0582 | |

*$P < 0·05$.          ***$P < 0·001$.

Analysis of variance of the square root transformations of the percentage lethal
figures up to the fifteenth day was carried out using orthogonal polynomials, and
the result of this analysis is given in Table 4.   The significant linear component of
the between-temperatures mean square shows that the *decrease* in mutation rate
with *rise* in temperature is significant and linear.   The difference between mutation
frequencies at different ages is highly significant, and is shown to have a significant
quadratic as well as a very highly significant linear component.   The quadratic
component is due to the initial decrease in mutation rate over the first few days
being followed by a rather constant rate, fluctuating around 0·1–0·2 per cent. approxi-
mately, depending on the temperature.   Finally, the absence of significant interaction
terms indicates that (i) the regression of mutation rate on temperature does not vary
with age, and (ii) the regression of mutation rate on age does not vary with tempera-
ture.   Figure 1 shows the fitted regression lines for the regression of mutation rate
on age of male for the three different temperatures used in this experiment.

IV. DISCUSSION

(a) *Heat Shock*

In Table 1 there is slight increase, though not significant, in the mutation rate
for the 1-hr treatment of larvae at 3 days of age.   This can readily be explained, even

if the figure were significantly higher than the other two larval treatments, because males arising from this particular treatment were mated in error as a group at 1–2 days of age instead of the usual procedure of 1–4 days old. This could have caused the slight rise in mutation rate in the 1-hr treatment.

The real difference between the 38°C replicates in Table 2 cannot be so easily explained. However, the higher rate in replicate 1 is undoubtedly due to the clumping of lethals in two particular treated males. The reason for this clumping is by no means clear. If males having more than, say, 10 per cent. lethals among their tested chromosomes are arbitrarily removed from the 38°C treatments, then the resulting mutation rates are approximately the same as control. It appears then that only a low proportion of males has sperm of high mutability when exposed to heat shock, and that the chance inclusion of two of these in replicate 1 is responsible
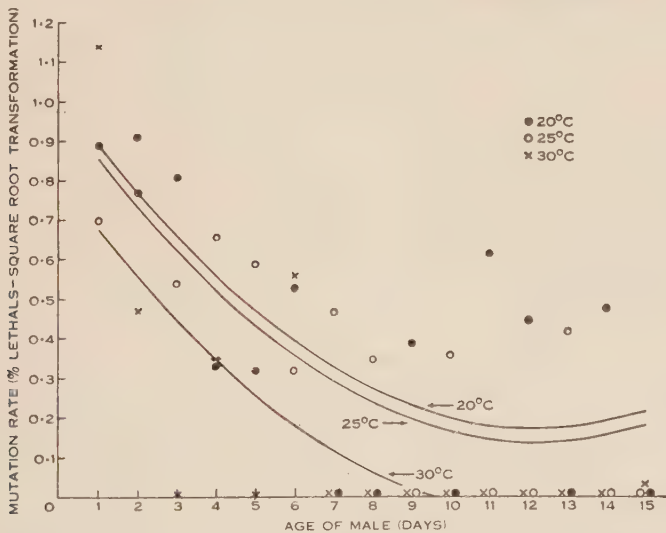


Fig. 1.—Fitted regression lines for the regression of mutation rate on age of male for the three different temperatures used.

for the difference between it and replicate 2. Such clumping of lethals is not a feature of a number of control series for determining spontaneous rates of adult males, at least within the author's experience, and response to heat shock might be simply a function of the number, if any, of sensitive males included in the treated samples.

The latter argument is supported by the results of heat shock at 40°C. Here again the higher figure for replicate 3 of the series is due to a particular male having seven lethals out of 31 tested $X$-chromosomes. If this male is excluded, then the resulting overall mutation rate at 40°C (0·04 per cent.) is significantly lower than controls ($\chi_1^2 = 9\cdot7$, $P < 0\cdot01$), as in the second replicate. In other words, shock of adult male sperm at extreme sublethal high temperature causes a drop in mutation rate for the majority of individuals but the usual increase in the 2 or 3 per cent. of sensitive flies. Reasons for such a decrease in mutation rate are not obvious. Two possibilities are that lethal-bearing sperm or even mutable males are killed by the

treatment, but in absence of general supporting evidence this must remain conjecture, especially as the sperm of one male survived the treatment to give 22 per cent. lethals.

It is not clear from Buchmann and Timofeeff-Ressovsky (1935, 1936), or from Plough (1941), whether their increases in mutation rate after heat shock of adult males were similarly due to clumping of lethals in a few sensitive individuals. The results reported here for adult males do not support their general conclusion that heat shock of adults necessarily increases the mutation rate. As indicated above, a number of conditions, including a specific temperature treatment, the presence and survival of highly mutable individuals, and the viability of lethal-bearing sperm apparently need to be fulfilled before an increase in mutation rate is obtained, as in replicate 1 at 38°C in this experiment. The possibility remains that the large number of mutations in the sensitive males might be the result of single or a few mutations occurring in spermatogonial cells. Since, however, males were test-mated immediately after treatment, it is unlikely that mutations occurring in spermatogonial stages in response to the treatment would have been represented so soon in the sperm sampled in this way. It is much more likely that the mutations obtained arose from the treatment of mature sperm, since these were the only treatments in which such clumping of lethals was found.

The present negative results on heat shock of larvae similarly do not support the finding of Buchmann and Timofeeff-Ressovsky. However, if a stage in the life cycle sensitive to heat shock is involved, it is quite possible that these workers, with their slightly wider range of larval ages during treatment covered the sensitive period, while the present experiment did not. A further explanation could be that the difference between the two sets of results is merely a strain difference, since it is generally known that different wild strains differ, at least in spontaneous mutability. Plough (1941) produced some evidence that only certain stocks with a high spontaneous mutation rate did not respond to heat shock. As the stock used here has an average spontaneous mutation rate, it is unlikely that its lack of response to heat in the shock larval stages is peculiar to this particular strain. It is far more likely that a sensitive stage has been missed in the present experiment, or that some other unknown variable was responsible for the increases obtained by the previous workers.

### (b) Temperature during Development

Previous studies had produced evidence to show that the mutation rate increases with increased temperature during development, and the data were interpreted in terms of van't Hoff's rules on the rates of reactions (Muller 1928; Timofeeff-Ressovsky 1935; Plough 1941). Increased mutation rate, in other words, was due to the increased rates of chemical reactions at the higher temperatures. The data reported here, however, in showing that mutation rate *decreases* with increasing temperature during the life cycle, renders the above interpretation untenable, and alternative explanations must be sought for the divergent results.

The present experiment made use of a mating scheme to ensure that, as far as possible, sperm were completely utilized as they matured. The results, therefore, are not confounded with environmental effects after maturation of sperm. Since Muller (1946) has reported that aging of mature sperm leads to an increase in mutation rate, and Byers (1954) has reported that this effect of aging is increased at

higher temperatures, it is possible that the earlier results of Muller, Timofeeff-Ressovsky, and Plough quoted above can be interpreted in terms of these environmental effects after maturation of sperm. The results reported here show that the developmental processes up to maturation of sperm do not respond to increased temperature by increased mutation rates. The significantly lower mutation rate with increased temperature can perhaps best be explained by the longer time period of development at lower temperatures, which would allow longer exposure, perhaps of sensitive stages, to other mutagenic agents. If the latter explanation is correct, then the possibility that increase in temperature is causing some increase in mutation rate cannot be ruled out. It is, however, obvious that such effects, if they exist, are completely over-shadowed by the indirect developmental effects of temperature in the opposite direction, and hence must be small in magnitude. Since it is difficult to postulate reasons for increased temperature *per se* being the cause of lower mutation rate, except perhaps in terms of a temperature-dependent balance between different chemical reactions, the above explanation based on developmental time appears to be a simple and reasonable one.

The results on effect of age in this experiment support the findings of Muller (1946) and other workers that the mutation rate in the sperm of the first few days is much higher than in later sperm, provided of course that sperm are used as they mature. The data also give some indication that the regressions (linear or quadratic components or both) of mutation rate on age vary with temperature, but this is not supported by the analysis of variance.

### (c) General

As indicated in the Introduction, one of the aims of these experiments was to relate responses obtained under heat shock treatments to those obtained when temperature during development was the variable. No obvious relationship can be observed from the data.

Temperature during development was varied only within a fairly narrow range (20 25–30°C) and responses obtained were apparently variations on a fairly random mutation process. No evidence of any highly mutable individuals was obtained, except perhaps for one male arising from the 30°C treatment. This individual scored three lethals out of 18 chromosomes tested, i.e. 17 per cent. lethals, on the first day, but no further lethals out of about 280 chromosomes tested on subsequent days up to the eleventh day. There is some indication, therefore, that this individual was sensitive to higher temperature in some pre-adult stage, but not after emergence, as were the particular males in the heat shock experiment. It must be remembered, however, that the sperm treated by heat shock were an older, and certainly more heterogeneous sample than sperm tested in the temperature-during-development experiment, unless the sperm present at the time of emergence of the adult are a relatively older sample than those newly matured sperm tested on the second and following days. If the latter point is a critical one then the initial response shown by the one sensitive individual at 30°C may bear some relationship to the type of response obtained with heat shock, but further evidence on all these aspects is needed before more definite conclusions can be drawn.

The general conclusion that mutation rate decreases with increased temperature during development, probably as an indirect effect of temperature on the length of the life cycle, follows readily from the data. Nevertheless, the weight of evidence in the literature supports the opposite view and the possible explanations of the difference are not entirely satisfactory. Further experiments on other strains along the lines of the design used here, and at the same time extending the range of temperatures used, should help to clarify the problem.

The experiment on heat shock in the larval stage covered only one well-defined period. Because of the negative correlation obtained between mutation rate and temperature during development, no evidence was obtained on whether any stages in the life cycle are more sensitive to heat shock. Evidence on this point will probably be provided only by heat shock treatments over a large number of short, well-defined periods in the life cycle.

## V. Acknowledgments

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# GROWTH OF THE MOUSE COAT

## VI. DISTRIBUTION AND NUMBER OF VIBRISSAE IN THE HOUSE MOUSE

### By R. B. Dun*

*Summary*

The vibrissae of 1-day-old mice were classified into major and minor groupings. The presence of many poorly developed vibrissae in the major groups made counting difficult. The minor groups were small isolated collections of large vibrissae. They were easily counted by the naked eye in 4- to 8-day-old mice.

The variation in the number of vibrissae in the minor groups of 3000 mice were investigated. The mice were from inbred and random-bred stocks showing normal coat development. Several discrete variations were found associated with the supra-orbital group. These abnormalities were not observed in the inbred lines. The number of inter-ramal vibrissae showed variation in all stocks except the inbred lines Aw101 and CBA. The other minor groups showed approximately one abnormality per 500 groups counted, with the exception of the postorbital vibrissa which was invariably present.

## I. Introduction

The distribution of certain of the facial vibrissae in the mouse has been described by Danforth (1925) and Figure 1, which shows the tactile hair arrangement on the head and fore limbs, is partly derived from his illustration. His observations were confirmed, in general, by Grüneberg (1943) although he describes the mystacial group as consisting of five horizontal rows and makes no mention of the vertical row E–F–G–H described by Danforth (1925). Davidson and Hardy (1952) examined the mystacial group and their findings support those of Danforth (1925). None of these authors mention the ulnar–carpal vibrissae (Beddard 1902) which occur on the fore limbs of the mouse.

Variation in the number of vibrissae was examined by Danforth (1925) using approximately 200 adult mice. He found no variation in the vertical row and the most posterior three vibrissae in the horizontal rows of the mystacial group. He noted one doubtful case in which a postorbital vibrissa appeared to be absent. The supra-orbital and postoral groups were slightly more variable showing 1 and 3 per cent. of aberrant scores respectively. Vibrissae were not counted at the inter-ramal and ulnar–carpal sites. The invariant nature of vibrissa number is particularly striking when consideration is given to the age of the mice which Danforth scored. Because of the cyclic activity of hair follicles, scores for vibrissa number in older mice could be falsely reduced by the shedding of fibres.

Grüneberg (1943) recorded the invariable presence of the postorbital vibrissa follicle in 140 normal mouse embryos. He also examined the postoral group and recorded the presence of two and, rarely, three follicles. He did not record specific observations on the remaining vibrissae.

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Davidson and Hardy (1952) described the number of vibrissae in the mystacial group but details of mice examined were not stated. Rows I and II each had four large vibrissae. Row III had five vibrissae while there were always at least six and eight vibrissae in rows IV and V respectively. Hardy (personal communication, 1956) indicated that in counts on approximately 100 young mice there was agreement with the earlier counts of Danforth (1925). In addition, the ulnar–carpal group showed 2 per cent. aberrant scores while the inter-ramals were highly variable (27 per cent. of groups with 2, 72 per cent. with 3, and 1 per cent. with 4 vibrissae).

Although the scoring methods used by these workers were varied and the number of mice examined was small, there is good agreement of results. In particular only one doubtful case of a missing postorbital vibrissa was observed. All other vibrissae showed a small amount of variation, the exception being the inter-ramals which were shown to be highly variable by Hardy.
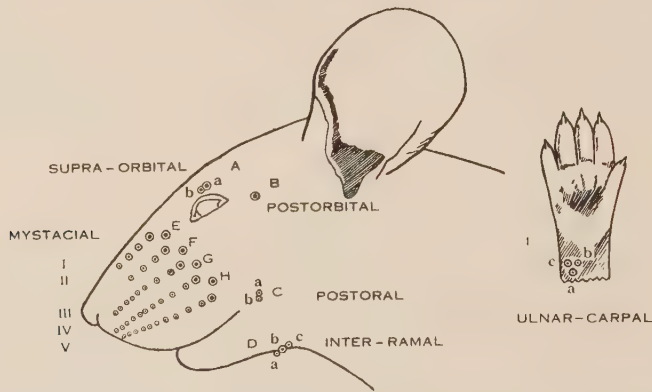


Fig. 1.—Head and distal part of right fore limb of the mouse showing
distribution of vibrissae.

Our interest in mouse vibrissae has arisen firstly from their low variability in normal mice, and secondly from the reduction in vibrissae which is produced by the pleiotropic genes crinkled (cr) (autosomal) (Falconer, Fraser, and King 1951), its sex-linked mimic tabby (Ta) (Falconer 1953), and ragged (Ra) (Carter and Phillips 1954). Waddington (1952) has shown that an invariant character, e.g. wing venation in Drosophila melanogaster, can be caused to vary by suitable treatment. Selection on such variability can be used to build up genetic effects which eventually make themselves felt in the absence of the treatment. We wished to see whether the variation introduced into the expression of a character by a major gene could be used in selection to change an invariant character eventually even in the absence of the gene.

A selection experiment was therefore planned, the aim being to influence vibrissa number by selection of genes which are unmasked by inclusion of the tabby gene in the selection stock. If these genes are identical or partially identical with the polygenic combinations which are responsible for the strongly buffered vibrissa development, such selection would eventually lead to a breakdown in the homoeostatic mechanism. Before the results of such an experiment could be evaluated, the normal variation in vibrissae would have to be known with greater accuracy than is possible

from an examination of the literature. This paper describes the examination of large numbers of mice in an attempt to measure the variation in vibrissa number between and within mouse strains. In addition, an examination was made of the entire vibrissa complement of the mouse in order to find the groups which could be most easily and accurately counted.

## II. MATERIALS AND METHODS

When making complete vibrissa counts, 1-day-old mice were fixed in formol-saline and examined with a dissection microscrope. An ocular micrometer was used for measurement of vibrissa length. Mice for this part of the observations were produced by crossing inbred line CBA with an albino stock.

TABLE 1
NUMBER OF VIBRISSAE IN MYSTACIAL ROWS III, IV, AND V

|  | Row III | Row IV | Row V |
|---|---|---|---|
| Number of rows counted | 306 | 306 | 306 |
| Mean number of vibrissae | 9·88 | 10·61 | 11·44 |
| Standard deviation | 0·719 | 0·653 | 0·595 |

The smaller groups of vibrissae can be accurately counted by naked eye under a strong light. An extensive examination was therefore made of the variation in these groups, using mice between 4 and 8 days of age. Mice examined were from the normally coated stocks maintained at the Animal Genetics Section, C.S.I.R.O. All mice born over the period March to August 1956 were examined and scored. Mice were from the following stocks:

(i) *Inbred Lines*—Aw101; CBA; $C_3H$; $C_{57}$; A. These lines are all 30 or more generations inbred by full sib mating.

(ii) *Selection Lines on Oestrogen Sensitivity.*—WI/OS and BI/OS are the original high and low selection lines. They were selected from a heterogeneous albino stock

WI/OS ⟨——WG/OS ——WR/OS    BI/OS ⟨——BG/OS ——BR/OS
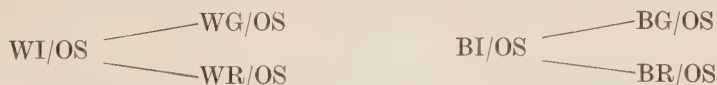
and have been separated for about 10 generations. WG/OS and WR/OS and BG/OS and BR/OS are sublines of WI/OS and BI/OS respectively. They have been separated from their parent lines for four generations. There are 40 matings in each line and inbreeding has been minimized.

(iii) *WOS.*—This is a general laboratory stock of albinos formed from BI/OS and A strain inbreds.

(iv) *LB.*—This stock has several segregating genes including pied and dilute.

## III. RESULTS AND DISCUSSION

The distribution of vibrissae in the mouse is illustrated in Figures 1 and 2. Size of dots is an indication of vibrissa size. Comparative lengths of vibrissae are presented in Figure 3. Observations are recorded below under group headings.

### (a) *Distribution of Vibrissae—Major Groups*

(i) *Mystacial*.—This group includes a vertical row E–F–G–H and horizontal rows I, II, III, IV, and V (Fig. 1). The vibrissae are arranged on each side of the snout, the horizontal rows starting from positions which alternate with the vibrissae of the vertical row.

Vibrissae E, F, G, and H, were present in all mice examined. Rows I and II usually had four vibrissae each as reported by Davidson and Hardy (1952) but in three out of 612 groups counted the most anterior vibrissa was missing. Rows III, IV, and V were much longer than reported by previous workers (see Table 1). As is shown in Figure 3 only the most posterior four vibrissae of each row were large. This is not so
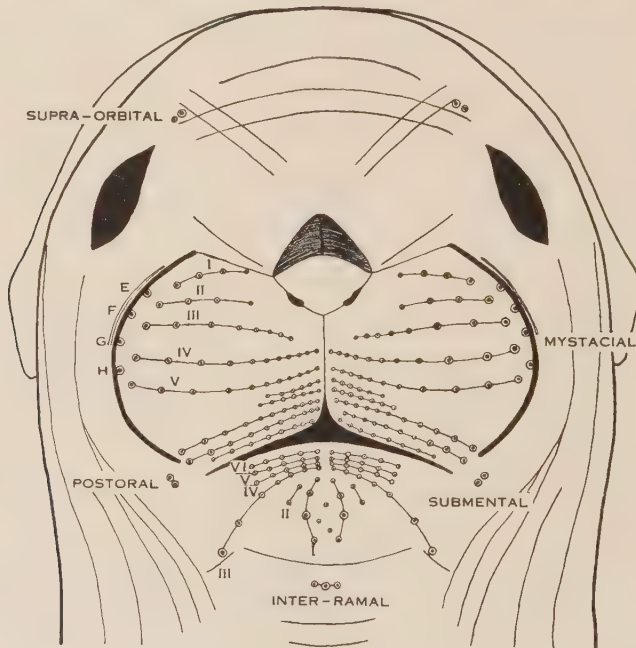


Fig. 2.—Anterior view of the head of a new-born mouse showing distribution of vibrissae.

obvious in the new-born mouse, where there is a smooth gradient from the longest posterior vibrissa to the shortest anterior vibrissa. The large number of small vibrissae makes these rows difficult to count with accuracy. Variation between mice is marked, as shown by the standard deviations in Table 1.

Below the main mystacial group are further small vibrissae (Fig. 2) arranged in horizontal rows which commence at the philtrum and run back parallel to the lips. Two to three short rows (5–8 vibrissae) commence beneath row V and these are followed by two to four longer rows (9–11 vibrissae) which extend to the lip commissures. Further short rows are present but the inturning of the upper lip limits observation.

(ii) *Submental*.—This group has not been previously described because the vibrissae are, in the main, small and tend to be obscured by coat hairs in older mice.

The pattern is of curved rows radiating from a point on the mid line ventral to the lower lip. The rows are symmetrical with the exception of three centrally placed vibrissae. Commencing from the mid line, row I has four to five vibrissae of which the second most posterior is large and conspicuous. Row II has two to three vibrissae. Row III extends laterally with six to seven vibrissae, the posterior vibrissa being very large. Above this row, several further rows, each of four to six small vibrissae can be seen.

Like the ventral rows of mystacials, the submental vibrissae are variable in number and difficult to count. The only vibrissae from the major groups which would be of value for an invariant selection score are mystacial rows I and II.
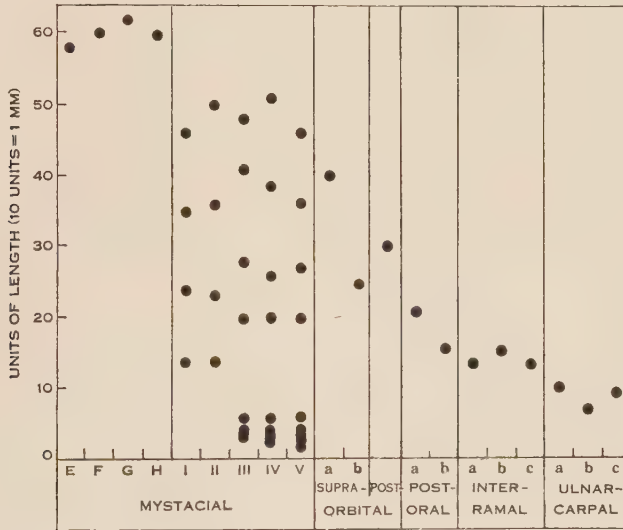


Fig. 3.—Comparative length of mouse vibrissae at 5 days of age.

### (b) Distribution of Vibrissae—Minor Groups

(i) *Supra-orbital.*—A, a and b (Fig. 1). These two vibrissae arise from a single tubercle placed above the eye. The large vibrissa a is postero-medially placed to its smaller companion b.

(ii) *Postorbital.*—B (Fig. 1). This is a single large vibrissa placed posterior to the eye.

(iii) *Postoral.*—C, a and b (Fig. 1). This group consists of two vibrissae, a (dorsal) and b (ventral), arising from a single tubercle which is found posterior to the lip commissures.

(iv) *Inter-ramal.*—D, a, b, and c (Fig. 1). The usual arrangement is a transverse row of three vibrissae centrally placed between the rami of the lower jaw.

(v) *Ulnar–carpal.*—I, a, b, and c (Fig. 1). These vibrissae arise from a single large tubercle on the lateral volar surface of the forearm just above the carpus. The

vibrissae are arranged at the corners of an equilateral triangle, the apex being proximal on the limb. Designation is *a*, proximal, *b*, medial distal, and *c*, lateral distal.

### (c) Variation in the Number of Vibrissae in the Minor Groups

(i) *Inbred Lines.*—Results are summarized in Table 2 and Figure 4. With the exception of the inter-ramals, group size is remarkably constant. Incidence of vibrissa deletions was of the order of one in 500 groups examined, the postorbital vibrissa being an exception in that it was invariably present. Apart from the inter-ramals, the only increased count was seen in a DBA mouse with a group of three postoral vibrissae.

TABLE 2

FREQUENCY DISTRIBUTION OF THE NUMBER OF VIBRISSAE IN SIX INBRED STRAINS OF MICE

| Strain | Supra-orbital | | Post-orbital | Postoral | | | Inter-ramal | | | | Ulnar-carpal | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of Vibrissae in Group | | | | | | | | | | | |
| | 1 | 2 | 1 | 1 | 2 | 3 | 1 | 2 | 3 | 4 | 2 | 3 |
| Aw101 | 1 | 459 | 460 | 1 | 459 | — | — | — | 230 | — | — | 460 |
| CBA | — | 496 | 496 | — | 496 | — | — | 3 | 245 | — | — | 496 |
| C₃H | — | 403 | 408 | 1 | 407 | — | — | 8 | 194 | 2 | 1 | 407 |
| C₅₇ | — | 324 | 324 | — | 324 | — | 1 | 22 | 137 | 2 | — | 324 |
| DBA | — | 308 | 308 | — | 307 | 1 | — | 56 | 98 | — | — | 308 |
| A | 1 | 231 | 232 | 1 | 231 | — | 1 | 62 | 47 | 6 | 9 | 223 |
| Total | 2 | 2226 | 2228 | 3 | 2224 | 1 | 2 | 151 | 951 | 10 | 10 | 2218 |
| Incidence (%) | 0·09 | 99·91 | 100·00 | 0·13 | 99·82 | 0·05 | 0·18 | 13·55 | 85·37 | 0·90 | 0·45 | 99·55 |

Figure 4 shows differences between inbred lines with respect to count variation in the inter-ramal group. No mice were detected in Aw101 with other than three inter-ramal vibrissae. The incidence of variation increased through CBA, C₃H, C₅₇, and DBA to a peak in A strain, where a score of three is seen in a minority of groups and possible counts range from one to four vibrissae.

Concomitant with the increase in inter-ramal variation, there is a marked drop in fertility. The bracketed numbers after the strain names in Figure 4 are the numbers of mice scored and as there are approximately equal numbers of matings in each line, this figure is a good index of fertility. The negative correlation between inter-ramal variation and fertility is clearly shown.

(ii) *Non-inbred Stocks.*—In these mice, both the supra-orbital and inter-ramal vibrissae show marked variation. Observations on these two groups are recorded in Table 3 and Figure 5. The postorbital vibrissa was again invariant while the postorals and ulnar–carpals showed about one reduced group per 500 scored.

*Supra-orbital score.*—Examination of Table 3 and Figure 5 reveals a very marked difference between BI/OS and WI/OS strains with regard to supra-orbital group size, the former showing a high incidence of groups with three vibrissae. The arrangement is shown as type *A* in Figure 6. The extra vibrissa was of a similar size to the shorter of the two normal vibrissae and was situated antero-medially on the same tubercle. In 36 per cent. of cases, groups were bilaterally affected. Two-thirds of the remaining mice show the "trio" grouping only on the right side.

Four mice were found in the line BG/OS where three vibrissae occurred above the eye, the arrangement differing in that the extra vibrissa was on a separate tubercle placed posterior to a normal supra-orbital group (Fig. 6, *B*).
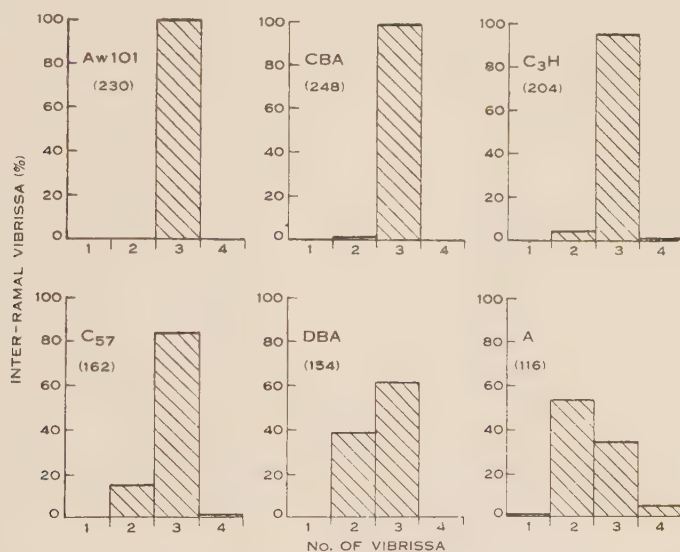


Fig. 4.—Frequency distribution of inter-ramal vibrissa count in six inbred mouse strains. Number of mice examined is shown in parenthesis for each strain.

*Inter-ramal score.*—Most stocks showed approximately 20 per cent. of groups with two vibrissae. The exception on the high variability extreme was LB, where the majority of inter-ramal groups had two vibrissae and there was almost as many with one as three vibrissae. BG/OS showed low variation with only 4 per cent. of reduced scores. Another point of interest was the frequent increase in inter-ramal group size in WI/OS and related strains. In WR/OS this was particularly well marked, two counts of five vibrissae being recorded.

Before concluding it is interesting to consider possible genetic models for the differences observed in the supra-orbital and inter-ramal groups. Considering the supra-orbital variants first, Table 4 shows parent–offspring comparison from BI/OS on the presence of trio groups (Type *A*, Fig. 6). The very large $\chi^2$ value indicates that this arrangement is highly heritable, possibly through a single gene showing 30–50 per

cent. penetrance when homozygous. Strong supporting evidence for this hypothesis is the complete absence of the trio group in the inbred lines and high frequency only in those lines directly derived from BI/OS. Because the effect of environment and modifiers is strong, it is not possible to test for the presence of a major gene until a stock selected for high penetrance is available. The inheritance of the BG/OS type $B$ arrangement is probably also governed by a major gene plus modifiers. The problem of test matings is even more acute in this case because penetrance is only 10 per cent. in matings between individuals showing the abnormality.

TABLE 3

FREQUENCY DISTRIBUTION OF THE NUMBER OF SUPRA-ORBITAL AND INTER-RAMAL VIBRISSAE IN NON-INBRED STRAINS OF MICE

| Strain | Supra-orbital | | | | Inter-ramal | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. of Vibrissae in Group | | | | | | | | |
| | 0 | 1 | 2 | 3 | 1 | 2 | 3 | 4 | 5 |
| WI/OS | — | 8 | 1636 | 2 | 1 | 157 | 660 | 5 | — |
| WG/OS | — | 1 | 438 | 1 | — | 46 | 172 | 2 | — |
| WR/OS | — | 2 | 458 | 2 | — | 49 | 170 | 10 | 2 |
| BI/OS | — | 6 | 1323 | 251 | — | 140 | 649 | 1 | — |
| BG/OS | 1 | 4 | 402 | 23 | — | 10 | 204 | 1 | — |
| BR/OS | — | 4 | 347 | 23 | — | 19 | 168 | — | — |
| WOS | — | 1 | 318 | 59 | — | 30 | 159 | — | — |
| LB | — | 1 | 105 | — | 9 | 31 | 13 | — | — |
| Total | 1 | 27 | 5027 | 361 | 10 | 482 | 2195 | 19 | 2 |
| Incidence (%) | 0·02 | 0·50 | 92·81 | 6·67 | 0·37 | 17·80 | 81·06 | 0·70 | 0·07 |

The inter-ramal variation is not easily explained. From examination of inbred line results, it was originally postulated that the inter-ramals were more sensitive to environmental fluctuation than the other vibrissae. In this case, the range of variation through the inbred lines could have been due to decreasing homoeostasis. This hypothesis was supported by the association between increasing inter-ramal variation and decreasing fertility.

No further support has been found for this hypothesis and a preferable explanation is provided by one of the forms of quasi-continuous inheritance listed by Grüneberg (1952). Experimental support was produced by crossing inbred lines, an intermediate level of inter-ramal count being obtained in the $F_1$. Selection high and low on inter-ramal vibrissa number was undertaken with mice from WR/OS. Selection changes have been marked in both directions indicating a high heritability. No fertility differences have been noted between the selection lines.

The other minor groups of vibrissae appear to be independant of the factors causing variation in inter-ramals in that they show no variation in any line, despite
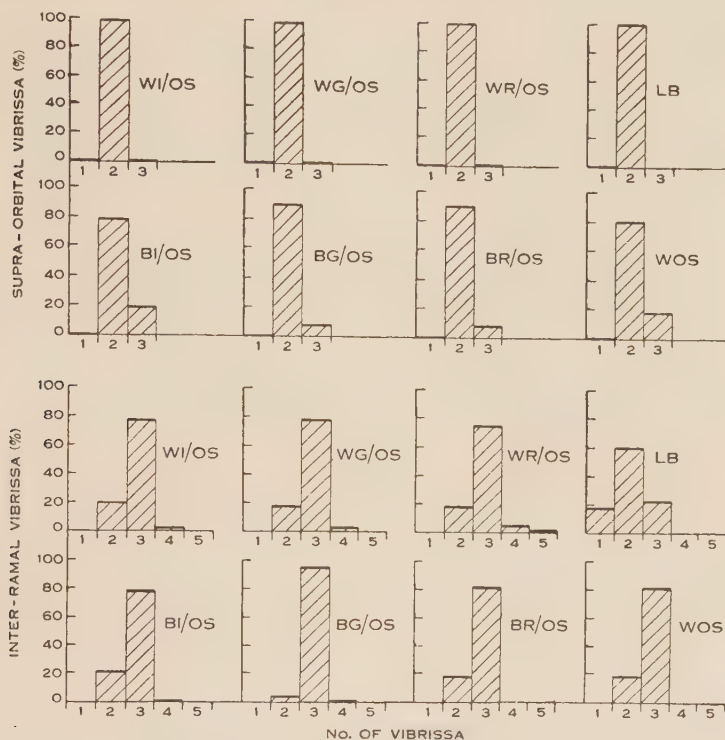


Fig. 5.—Strain differences in vibrissa counts of supra-orbital and inter-ramal groups (non-inbred stocks).

the range of inter-ramal expression.  Again in BI/OS, penetrance of the trio supra-orbital group was not correlated with inter-ramal vibrissa count.  Further support for



Fig. 6.—A, supra-orbital group with three vibrissae;  B, an extra vibrissae behind the normal supra-orbital group.

independance was obtained from the inter-ramal selection experiment, where although the two lines are widely separated on inter-ramal count, there are no correlated changes in the rest of the vibrissa complement.

## IV. Conclusions

Variations in the number of vibrissae in the minor groups of normal mice, were restricted to the following cases:

(i) Marked variation in the inter-ramal group with counts varying from one to five vibrissae. There were large strain differences which were readily explainable if the variation was governed by polygenic inheritance. Two inbred lines, Aw101 and CBA, were exceptional in that they showed practically no variation at the inter-ramal site.

(ii) Increase in the supra-orbital count from two to three vibrissae. This change was strain delimited and could be caused by a major gene.

(iii) Presence of an accessory supra-orbital vibrissa. This was again strain delimited and was probably associated with a major gene.

(iv) Rare vibrissa deletions occurring in all groups about once in 500 groups scored. The postorbital vibrissa was an exception in that it was invariably present in the 3000 mice examined.

TABLE 4

COMPARISON OF PROGENY FROM MATINGS WITH DIFFERENT SUPRA-ORBITAL SCORE COMBINATIONS

N indicates a normal score of two vibrissae on each side, and T a score of three vibrissae on each side, or three on one side and two on the other.   $\chi^2 = 58.86$,   d.f. = 3,   $P < 0.01$

| No. of Supra-orbital Vibrissae | Parent Combinations | | | | Total |
|---|---|---|---|---|---|
| | N♂ × N♀ | N♂ × T♀ | T♂ × N♀ | T♂ × T♀ | |
| N | 470 | 29 | 85 | 29 | 613 |
| T | 85 | 25 | 52 | 18 | 180 |
| Total | 555 | 54 | 137 | 47 | 793 |

Thus the development of mouse vibrissae is very strongly canalized. This is particularly the case in the inbred lines Aw101 and CBA and they would therefore be the best mice to use in the formation of a selection stock for an attempt to break down vibrissa development. In the evaluation of such an experiment, absence of any vibrissae from normal mice would lead one to suspect a change in the basic genotype and, in particular, absence of a postorbital vibrissa would be excellent evidence of such an upset.

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# THE QUANTITATIVE DETERMINATION OF THE IODO-AMINO ACIDS OF THYROID TISSUE

By T. H. Kennedy*

*Summary*

The ability of three different pancreatin preparations to liberate iodo-amino acids from thyroid tissue has been studied. Two of the pancreatin preparations, while freeing substantial amounts of the iodotyrosines, released only a part of the thyroxine.

A method of sodium hydroxide hydrolysis is described which produces less destruction of organic iodine-containing compounds, with reduced formation of iodide and identifiable artefacts.

The third pancreatin, a total extract of pig pancreas, gave thyroxine values in good agreement with those obtained by the modified alkaline method.

Most hydrolysates were analysed both by column and paper chromatographic methods, and the similarity of the values obtained indicates that both methods may be regarded as satisfactory.

## I. Introduction

The classical methods of alkaline hydrolysis of thyroid tissue or thyroglobulin as a preliminary to the determination of the iodinated amino acids have been discarded in recent years in favour of enzymic methods although little direct comparison of the two methods has been made. Braasch, Flock, and Albert (1954) have compared the action of trypsin and sodium hydroxide on human thyroid tissue, and concluded, on the bases of apparent incompleteness of hydrolysis and high iodide formation, that sodium hydroxide was inferior to tryspin, although in only one case was tissue from the same patient hydrolysed by both methods. Furthermore five samples of tissue from the same source gave, after trypsin hydrolysis, thyroxine values of 4–26 per cent. and di-iodotyrosine values of 17–36 per cent. of total iodine (two of the determinations gave thyroxine values of 20 and 26 per cent. and di-iodotyrosine values of 36 and 36 per cent. of total iodine). This wide range of values, for which no explanation is apparent, would not justify the use of tryptic hydrolysis as part of a quantitative procedure.

In the course of another investigation it was found that sodium hydroxide liberated nine times as much thyroxine from a rat thyroid homogenate as did a commercial trypsin preparation. The chromatographic system used was *tert.*-pentanol–ammonia in which the artefacts produced by the action of alkali on the iodinated tyrosines are separated from thyroxine (Stanley 1953; Kennedy 1957). This finding has led to an investigation of the ability of some different pancreatin preparations to release the iodo-amino acids from thyroglobulin. The amounts liberated have been compared with the amounts liberated by a modified sodium hydroxide hydrolysis.

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## II. MATERIALS AND METHODS

Rats were killed 8–96 hr after the injection of $^{131}I$ the dose of which was calculated so that the thyroids contained not more than 7 $\mu c$ at the time of maximum uptake. Thyroids were removed immediately after death of the rat and placed in a weighing bottle in ice. After weighing, the pooled glands were ground in a small mortar, previously cooled in ice, with 0·1 ml of water, 10–20 mg of glass powder, and 200 $\mu g$ of methyl thiouracil or thiourea. This addition was made to prevent artefact formation during the process of homogenizing and dividing for analysis (Taurog, Potter, and Chaikoff 1955). The suspension was then diluted with water to contain 50–100 mg tissue per ml.

In some experiments, the iodide and free iodo-amino acids of the gland were removed by five extractions with an equal volume of $n$-butanol after dilution with an equal volume of water and adjustment of the pH to about 2·5 with 0·1N HCl. Another 200 $\mu g$ of methyl thiouracil or thiourea was added to the aqueous phase and precipitated protein, which were then dried from the frozen state after removal of butanol by vacuum distillation at 30°C. When required, the dry residue was taken up in an appropriate amount of water, adjusted to pH 8–8·3 with 0·1N NaOH, and the suspension divided for enzyme and sodium hydroxide hydrolysis.

### (a) Sodium Hydroxide Hydrolysis

The thyroid homogenate containing up to 100 mg tissue was placed in a tube 8–10 mm in internal diameter, made 2N with respect to sodium hydroxide by the addition of 10N reagent, and then diluted with 2N NaOH to 4–5 ml. A further addition of about 100 $\mu g$ of methyl thiouracil or thiourea was made, and after thorough degassing at the water pump, the tube was sealed under vacuum. The sealed tubes were heated in an oven at 98–100°C for 16–18 hr.

In early experiments the hydrolysate was acidified to pH 2 with hydrochloric acid and extracted repeatedly with an equal volume of $n$-butanol until less than 5 per cent. of the total $^{131}I$ was left in the aqueous phase. Concentration of the pooled butanol extracts at 30°C gave a residue which contained too much salt for satisfactory paper chromatograms to be run and it was found necessary to dilute the concentrate with water and repeat the butanol extraction. A simpler method of removing the excess sodium ion was to run the hydrolysate through a column of the cation-exchange resin "IRC 50" in the acid form. The column was then washed with 1·0N $NH_4OH$ until the residual $^{131}I$ was less than 1 per cent. of the total. The effluent was concentrated in vacuo at 30°C nearly to dryness, when the solution usually became semi-solid with colloidal silica. The solid was extracted two or three times with 1 ml 90 per cent. methanol–water (v/v) 0·1N with respect to ammonia, and filtered through a small cotton-wool plug. The methanol solution was taken to dryness in vacuo.

### (b) Enzyme Hydrolysis

Three different preparations of pancreatin were used, two of which, A and B, were commercial preparations. The third, C, was prepared by extracting minced fresh pig pancreas with two volumes of water overnight. After centrifuging, the

supernatant was dried from the frozen state. The dry powder was extracted twice with anhydrous ether, dried in air, taken up in a smaller amount of water, centrifuged, and dried again from the frozen state.

About 5 mg of pancreatin was added to 1 ml of homogenate containing 50–100 mg thyroid tissue, the pH adjusted to 8–8·3 with sodium hydroxide, and the digest kept at 37°C. Where aliquots were taken at intervals they were frozen until analysed. Some enzyme digests were dried from the frozen state before analysis.

In some experiments the homogenate was heated for 2–3 min in a boiling water-bath to inactivate thyroid enzymes, particularly the dehalogenase (Roche *et al.* 1953) which it was thought might be contributing to the inorganic iodide formed during hydrolysis.

TABLE 1

APPROXIMATE $R_F$ VALUES IN *tert.*-PENTANOL–AMMONIA AND *tert.*-PENTANOL–ACETIC ACID SYSTEMS

| Compound | $R_F$ in: | |
|---|---|---|
| | *tert.*-Pentanol–Ammonia | *tert.*-Pentanol–Acetic Acid |
| Thyroxine | 0·30 | ⎱ 0·85 ⎰ |
| 3,5,3′-Tri-iodothyronine | 0·50 | |
| 3,5-Di-iodotyrosine | <0·1 | 0·45 |
| Mono-iodotyrosine | <0·1 | 0·37 |
| Iodide | 0·15 | 0·11 |
| 3-Iodo-4-hydroxybenzaldehyde | 0·50 | ⎱ 0·85 ⎰ |
| 3,5-Di-iodo-4-hydroxybenzaldehyde | 0·65 | |

### (c)  *Analysis of Hydrolysates*

(i) *Column Chromatograms.*—Dry residues were taken up in 2·5N NaOH, and enzyme digests which were not dried were made 2·5N by addition of 10N NaOH, kieselguhr and a little mobile phase added, and the slurry packed on top of a prepared column. Columns were run as described by Kennedy and Purves (1956) with a modified solvent system, the *tert.*-butanol being followed by *tert.*-butanol–*n*-propanol mixtures instead of the *n*-propanol–*cyclo*hexane mixture previously described. Added carrier compounds were located as previously described and the activity of each fraction measured by γ-counting. Fractions were pooled for final counting on the basis of radioactivity and carrier distribution. The combined fractions were evaporated to 3–4 ml before counting.

(ii) *Paper Chromatograms.*—Whatman No. 1 paper, 10 × 40 cm was used. The paper was cut to give five parallel strips separated by 2-mm spaces.

The solvent systems used were *tert.*-pentanol–2N $NH_4OH$ (1 : 1 v/v) (Gleason 1955) for measurement of thyroxine, 3,5,3′-tri-iodothyronine, and iodide, and *tert.*-pentanol–acetic acid–water (9 : 1 : 10 v/v) for measurement of iodide and the two iodinated tyrosines. The aqueous phase of the acid system was saturated before each run with hydrogen sulphide. Approximate $R_F$ values are given in Table 1.

Iodide determinations obtained from the acetic acid system were in close agreement with those from the ammonia system, and only the latter are given.

TABLE 2

DETERIORATION OF PANCREATIN A WITH TIME

| Hydrolysis | Chromatographic Analysis | Iodo-amino Acids as Percentage of Total Thyroid [131]I | | | | |
|---|---|---|---|---|---|---|
| | | $T_4$* | $T_3$* | DIT* | MIT* | Iodide |
| Early experiments: | | | | | | |
| Pancreatin A, 48 hr | Paper | 6·6 | 1·2 | 19·0 | 36·8 | 3·4 |
| | Column | 6·7 | 1·4 | 18·9 | 32·8 | 3·6 |
| Experiments 6 months later | | | | | | |
| Pancreatin A, 96 hr | Paper | 0·8 | 0·0 | 21·2 | 33·8 | 4·6 |
| 2N NaOH, 16 hr† | Paper | 7·3 | 1·4 | 26·2 | 25·0 | 24·7 |

*In this and subsequent tables, $T_4$, $T_3$, DIT, and MIT refer to thyroxine, 3,5,3'-tri-iodothyronine, 3,5-di-iodotyrosine, and mono-iodotyrosine respectively.

†Old method of NaOH hydrolysis.

Dry residues from the hydrolysates were taken up in 0·1N $NH_4OH$ and, after thorough grinding with a glass rod, 2–10 $\mu$l of the suspension applied in a band across

TABLE 3

COMPARISON OF AMOUNTS OF IODO-AMINO ACIDS LIBERATED FROM A RAT THYROID HOMOGENATE BY PANCREATINS B AND C AND BY SODIUM HYDROXIDE

| Hydrolysis | Time (hr) | Chromatographic Analysis | Iodo-amino Acids as Percentage of Total Thyroid [131]I | | | | |
|---|---|---|---|---|---|---|---|
| | | | $T_4$ | $T_3$ | DIT | MIT | Iodide |
| Pancreatin B | 24 | Paper | 4·4 | 0·7 | 27·5 | 29·4 | 4·2 |
| | 48 | Paper | 5·7 | 0·7 | 29·1 | 26·3 | 4·2 |
| Pancreatin C | 24 | Paper | 8·0 | 1·2 | 43·0 | 25·0 | 3·9 |
| | 48 | Paper | 8·6 | 1·4 | 41·8 | 25·5 | 4·6 |
| | 48 | Paper | 7·9 | 1·5 | 39·8 | 24·0 | 4·4 |
| | 48 | Column | 8·2 | 1·2 | 42·6 | 20·4 | 3·9 |
| 2N NaOH | 16 | Paper | 12·4 | 1·6 | 37·4 | 16·1 | 8·1 |
| | 16 | Column | 10·1 | 2·3 | 40·0 | 18·6 | 8·3 |

the 2-cm strip. Enzyme digests were applied directly to the paper in the same quantity. After drying, the papers were left in the tank for 1–2 hr before application of the

TABLE 4

COMPARISON OF AMOUNTS OF IODO-AMINO ACIDS LIBERATED FROM DIFFERENT THYROID HOMOGENATES BY PANCREATIN C AND SODIUM HYDROXIDE

| Homogenate | Hydrolysis | Time (hr) | Chromatographic Analysis | Iodo-amino Acids as Percentage of Total Thyroid $^{131}$I | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | $T_4$ | $T_3$ | DIT | MIT | Iodide |
| 1 | Pancreatin C | 24 | Paper | 14·2 | 2·1 | 33·6 | 30·8 | 3·1 |
| | | 48 | Paper | 15·6 | 2·4 | 35·9 | 29·3 | 3·5 |
| | | 96 | Paper | 14·6 | 3·1 | 38·6 | 27·6 | 8·5 |
| | | 96 | Paper | 14·1 | 2·9 | 37·8 | 28·6 | 8·1 |
| | | 96 | Column | 13·2 | 3·6 | 37·5 | 27·0 | 7·3 |
| | 2N NaOH | 17 | Paper | 14·7 | 1·9 | 27·2 | 18·6 | 8·2 |
| | 2N NaOH | 17 | Paper | 13·2 | 1·9 | 26·3 | 19·3 | 7·2 |
| 2 | Pancreatin C | 96 | Paper | 11·9 | 6·6 | 16·3 | 32·8 | 4·9 |
| | | 96 | Paper | 12·3 | 6·5 | 15·3 | 32·7 | 5·5 |
| | | 96 | Column | 13·5 | 8·7 | 13·6 | 34·3 | 4·7 |
| | 2N NaOH | 16 | Paper | 8·2 | 7·3 | 18·8 | 34·0 | 12·3 |
| 3 | Pancreatin C | 48 | Paper | 12·3 | 2·0 | 39·4 | 18·0 | 9·6 |
| | | 48 | Paper | 10·8 | 2·5 | 41·1 | 18·2 | 10·9 |
| | 2N NaOH | 16 | Paper | 11·7 | 2·1 | 28·4 | 12·3 | 15·5 |

mobile phase. The chromatograms were run for 16–20 hr after which time the solvent front was about 25 cm from the origin. Mixtures of known substances were run on parallel strips, and all films were autographed on Ilford X-ray paper to check the separation. The papers were cut up for counting on the basis of the autograph and the separate pieces counted under a thin end-window tube (General Electric Co. 2B7).

## III. RESULTS

One sample of commercial pancreatin, A, liberated considerable amounts of 3, 5-di-iodotyrosine and mono-iodotyrosine from thyroid tissue but only 1 per cent. of the total iodine was present as thyroxine at 48 hr. Further addition of enzyme and readjustment of the pH did not produce any substantial alteration in the
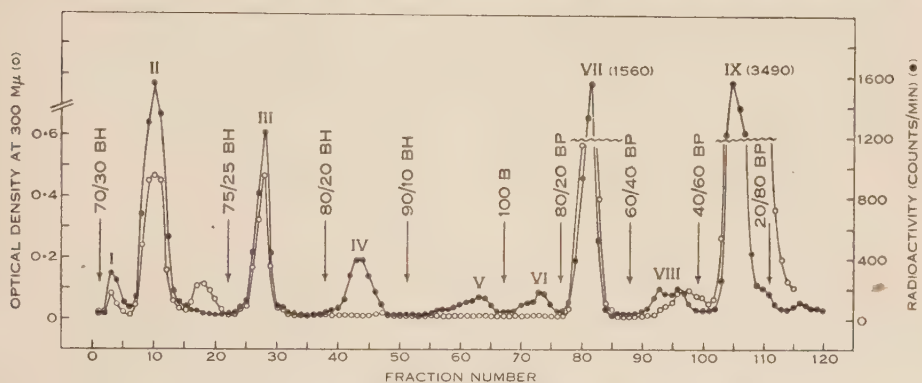


Fig. 1.—Column chromatogram of pancreatin digest of rat thyroid homogenate 2. Solvent changes were made at the points indicated. BH, *tert.*-butanol-*cyclo*hexane (v/v) mixture; BP, *tert.*-butanol-*n*-propanol (v/v) mixture. The radioactivity of the fractions is shown by the solid circles, and the optical density at 300 mμ, giving the distribution of the carriers, is shown by open circles. Identified peaks are: II, thyroxine; III, 3,5,3'-tri-iodothyronine; IV, iodide; VII, 3,5-di-iodotyrosine; and IX, mono-iodotyrosine. The small absorption peak between II and III is the methyl thiouracil added to the digest. Values in parenthesis give the maximum radioactivity for the respective peaks. The companion ultraviolet absorption peaks were off the scale.

proportion of these amino acids. Subsequent alkaline hydrolysis of this digest liberated 7·3 per cent. of the total iodine as thyroxine. In experiments carried out some months earlier this pancreatin had given much greater amounts of thyroxine from similar rat thyroids, although the results had not been compared with those of alkaline hydrolysis. The results are given in Table 2.

The amounts of the different iodo-amino acids liberated by the enzyme preparations B and C from the same thyroid homogenate are shown in Table 3, together with the amounts liberated by modified sodium hydroxide hydrolysis. Further comparison of the values obtained by the action of enzyme preparation C and sodium hydroxide on different thyroid homogenates is given in Table 4.

The method of sodium hydroxide hydrolysis used here differs in three respects from that used previously (i) in the presence of small quantities of thiourea or methyl thiouracil, (ii) in the reduction of the amount of oxygen present, and (iii) in the increased ratio of sodium hydroxide solution to protein. No attempt has been

made to assess the influence of these factors individually but the system described did not produce identifiable artefacts, and the amount of iodide formed was less than that frequently encountered with the old method where the volume of sodium hydroxide was less and air was not excluded.

Pooled concentrated fractions from the column analysis of homogenate 2, the effluent curve of which is shown in Figure 1, were run through an "IRC 50" column which was washed with dilute ammonia as described earlier, and the effluents dried from the frozen state. The residues were taken up in 0·1 ml of 0·1N $NH_4OH$ and aliquots run on paper with the two solvent systems described. The papers were autographed and then sprayed to locate the carriers. The thyroxine, tri-iodothyronine, iodide, di-iodotyrosine, and mono-iodotyrosine peaks all gave a strong radioactive band corresponding in position with the carrier, in both solvent systems. In all the iodo-amino acid fractions faint iodide bands were also present. The first column peak, fractions 1–6, when run on paper showed faint bands on the origin, and in the iodide position in both solvents. There were no bands corresponding to the iodo-hydroxybenzaldehydes (Kennedy 1957). No identifiable bands, other than traces of iodide, were found in the paper runs of the other column peaks.

## IV. Discussion

The chief objections to the conventional method of alkaline hydrolysis have been the production of artefacts and the liberation of considerable amounts of iodide. The artefacts in some solvent systems run in the same position as thyroxine and lead to high values. Thus Roche, Michel, and Volpert (1954) concluded that all of the apparent thyroxine found in an alkaline hydrolysate of casein iodinated enzymically by the procedure of Keston (1944) was an artefact as it was not produced during enzyme hydrolysis. Taurog et al. (1955) found that trypsin hydrolysis of sheep thyroid slices gave thyroxine values lower than those obtained in earlier work where sodium hydroxide was used. This error may be avoided by the use of a chromato-graphic system which separates the artefacts from thyroxine, but the very presence of these substances, together with inorganic iodide, which is frequently 20–30 per cent. of the total iodine, is evidence of destruction during the hydrolytic procedure. Using such systems very low thyroxine values, amounting to about 1 per cent. of the total iodine, have been frequently obtained, particularly in hyperplastic thyroids where the total iodine content is low (Kennedy and Purves 1956).

The modified sodium hydroxide hydrolysis described here reduces the magnitude of some of these errors. The iodide found is considerably less than that produced by the conventional method although in general it is higher than that found in enzymic hydrolysates. The thyroxine values, in most cases, are in reasonable agreement with those found after hydrolysis by a total pancreas extract preparation. In addition, in no instance has any detectable amount of either of the iodohydroxy-benzaldehydes been encountered (Kennedy 1957).

Two of the enzyme preparations used liberated mono- and di-iodotyrosine and thyroxine, and in the acetic acid–pentanol system only traces of radioactivity were left on the origin in the thyroglobulin position. The amount of thyroxine, however, was less than that obtained after alkaline hydrolysis. The third enzyme

preparation gave thyroxine values in good agreement with those obtained by the modified sodium hydroxide method and there was also a reasonable agreement between the figures obtained from either hydrolysate when analysed by two different chromatographic systems. The values for the iodinated tyrosines after chemical hydrolysis were sometimes much lower than those after enzymic hydrolysis. In this case the latter values are to be preferred since, for a given enzymic hydrolysate, the different analytical systems gave substantially the same result.

Trypsin, like sodium hydroxide, liberated variable but significant amounts of iodide from thyroid tissue. This was found in enzymic hydrolysates of thyroglobulin which had been previously extracted with butanol, as well as in those which had been heated to destroy the thyroid dehalogenase system. Most of this iodide must be formed during the hydrolysis since different methods of analysis gave closely similar results. This would be unlikely if it were formed during the separation procedures. This iodide probably arises by spontaneous breakdown in solution of the major constituents, as these fractions from column analyses always contain some iodide when they are run on another column or on paper.

The failure of some trypsin preparations to liberate all of the thyroxine, although substantial amounts of the iodo-tyrosines are produced, may be responsible for some of the very low thyroxine values reported in the literature, e.g. the value of 0·45 per cent. total iodine reported by Stanley (1956) for normal human thyroglobulin.

It is suggested that enzyme preparations should be checked from time to time for their ability to release thyroxine, by comparison either with the alkaline method described here or with another enzyme preparation.

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# FUNGAL CELLULASES

## VIII. FURTHER OBSERVATIONS ON THE β-GLUCOSIDASE OF STACHYBOTRYS ATRA

### By M. A. Jermyn*

*Summary*

A number of experiments have been carried out which were designed to throw further light on the specificities of the "substrate" and "acceptor" centres of the β-glucosidase of *S. atra*. The type 2 activator, glycerol, has been shown to be an acceptor for a glucosyl residue transferred from *p*-nitrophenyl β-glucoside. *p*-Nitrophenyl 6-*O*-*p*-toluenesulphonyl-, 6-*O*-methyl-, and 3-*O*-methyl-β-D-glucopyranosides are all substrates for the β-glucosidase. The implications of the observations for the theory of the action of the enzyme are discussed.

## I. Introduction

The sixth paper of this series (Jermyn 1955*b*) left a number of questions about the specificity of the β-glucosidase of *Stachybotrys atra* unanswered and this paper records some attempts to produce the required answers. The material here presented does not form a logical whole but, since the author is unlikely to return again to the study of this enzyme in the near future, he feels it best to publish the results of a number of small investigations under a single heading. Some of the experimental results obtained, especially those concerned with the products of hydrolysis in the presence of various inhibitors, have already been discussed at length elsewhere (Jermyn 1957*a*), and the relevant portions of the present paper may be looked on as providing experimental justification for the statements made there.

## II. Methods and Materials

The methods used in this work do not differ materially from those used previously (Jermyn 1955*a*, 1955*b*). Any small deviations will be noted at the appropriate point.

Many of the substances used as potential inhibitors or substrates have been synthesized for the first time and their synthesis is recorded elsewhere (Jermyn 1957*b*). D-Threose and D-erythrose were synthesized according to Perlin and Brice (1955), 2-*O*-methylglucose according to Hodge and Rist (1952), and 6-*O*-methylglucose according to Bell (1936). 3-*O*-methylglucose, 2-deoxyglucose and D-rhamnose were commercial samples.

## III. Hydrolysis of Thioglucosides

The β-glucosidase of *S. atra* appears to be unique amongst those so far studied in hydrolysing phenyl β-thioglucoside, which is also a competitive inhibitor of the enzymic hydrolysis of *p*-nitrophenyl β-glucoside. However, the activity against the thioglucoside is low and the results show great experimental scatter, perhaps owing to erratic oxidation of the liberated mercaptan in dilute solution. The hypothesis that

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the hydrolysis of the $O$- and $S$-glucosides was due to the same enzyme was checked by showing that the Michaelis constant ($K_m$) of the $S$-glucoside as a substrate was approximately the same as its inhibitor constant ($K_i$) as a competitive inhibitor. For the reason given above the determination of $K_m$ involved a long and uncertain
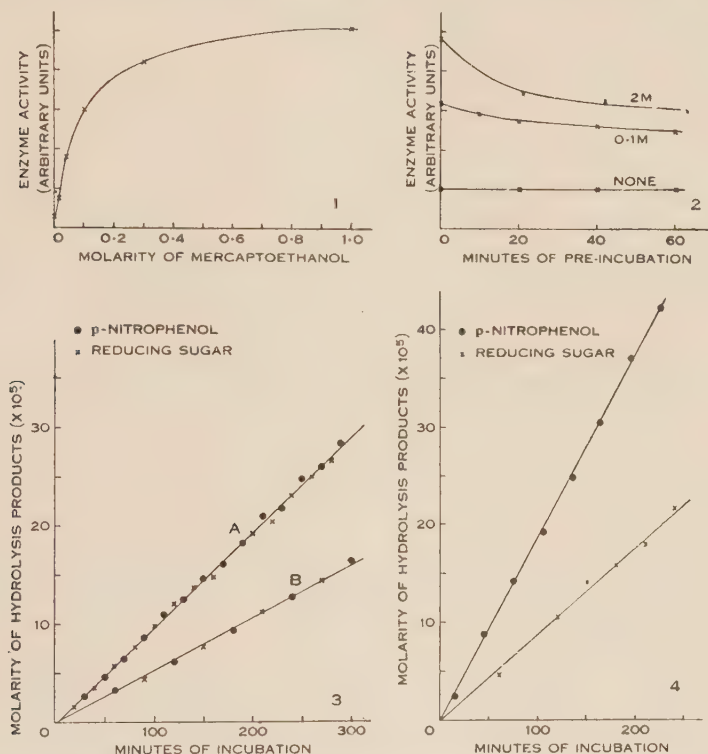


Fig. 1.—Effect of mercaptoethanol concentration on the hydrolysis of $p$-nitrophenyl β-D-glucoside ($10^{-3}$M) by the $S.\ atra$ β-glucosidase for 20 min at 28°C in McIlvaine sodium phosphate–citric acid buffer (pH 5·0).

Fig. 2.—Effect of pre-incubation of $S.\ atra$ β-glucosidase at 28°C with the indicated concentrations of mercaptoethanol on the enzymic activity determined under the conditions of Figure 1.

Fig. 3.—Hydrolysis of $p$-nitrophenyl β-D-glucoside ($10^{-3}$M) by the $S.\ atra$ β-glucosidase at 28°C and pH 5·0, $A$, alone and $B$, in the presence of $10^{-2}$M phenyl α-cellobioside. Both $p$-nitrophenol and reducing sugar concentrations are indicated.

Fig. 4.—Hydrolysis of $p$-nitrophenyl β-D-glucoside ($10^{-3}$M) by the $S.\ atra$ β-glucosidase at 28°C and pH 5·0 in the presence of $10^{-1}$M glycerol. Both $p$-nitrophenol and reducing sugar concentrations are indicated.

extrapolation and it therefore seemed advisable to cross-check the hypothesis by studying the reverse case—inhibition of the hydrolysis of $p$-nitrophenyl β-thioglucoside by phenyl β-glucoside.

$p$-Nitrophenyl β-thioglucoside on synthesis proved to be very difficult to use as a substrate. In solution at pH 5–9 it decomposed rapidly and rough experiments showed that at pH 5 and 28°C it broke down by the "alkaline hydrolysis" mechanism

$10^4$–$10^6$ times as fast as the $O$-glucoside. The blanks for the experiments with the enzyme were thus very high, and although this difficulty could be minimized by using freshly prepared solutions and suitably diluting them, only fairly high enzymic activities would have been demonstrable. A further complication appeared when an attempt was made to demonstrate the actual existence of such activities. The incubation mixture containing enzyme and substrate was found to have a lower optical density than the blank containing substrate alone.

The obvious hypothesis to account for this observation was to suppose that the $p$-nitrothiophenol originally present in the solution was reacting with the enzyme protein. Youatt (personal communication) informs me that he observed a similar "negative activity" in the reaction between trypsin and diethyl-$S$-$p$-nitrophenyl phosphorothiolate which should phosphorylate trypsin with the liberation of $p$-nitrothiophenol. If such reaction with proteins is a general property of $p$-nitrothiophenol it should be inhibited by the addition of sufficient quantities of other thiol compounds.

Additions of cysteine, glutathione, and thioglycollic acid failed to affect the $p$-nitrothiophenol–protein reaction at any concentration. However, concentrations of mercaptoethanol higher than about 0·5M completely inhibited this reaction, but in the system enzyme–substrate–1M mercaptoethanol it was impossible to demonstrate any enzymic activity. That this observation was not due to inhibition of the enzyme by mercaptoethanol but to an inherently low enzyme activity was demonstrated by the observations set forth in Section IV.

The negative result of the present work leaves the earlier observations (Jermyn 1955b) still the only evidence for the hydrolysis of thioglucosides by the $S$. $atra$ $\beta$-glucosidase.

## IV. Effects of Mercaptoethanol

Mercaptoethanol, tested for its effect on the enzymic hydrolysis of $p$-nitrophenyl $\beta$-glucoside under the standard conditions, where active agent and substrate were mixed together in solution and the enzyme added, was found to be an activator (Fig. 1).

Since activation by thiols is often time-dependent the enzyme and the mercaptoethanol were mixed and incubated together for varying periods before addition to the substrate (Fig. 2). The results of this experiment seem to show an immediate activation, followed by a slow inactivation, the rate of which not only increases with increasing mercaptoethanol concentration but also falls off with time. However, since the enzyme activity in the presence of mercaptoethanol at zero time is really measured after 20 min further incubation in the presence of mercaptoethanol at one-fifth of the original concentration, another experiment was devised to eliminate any effects due to this further incubation. Enzyme at a suitable dilution was incubated alone or with mercaptoethanol for 10 min. These enzyme samples were then added to standard substrate mixtures with and without mercaptoethanol respectively and incubated for the standard period (20 min). The results are set out in Table 1. The independence of the activating and inactivating effects and the increase in the rate of the latter with mercaptoethanol concentration are clearly demonstrated.

The activation with mercaptoethanol is apparently an ordinary type 2 activation (Jermyn 1955b, 1957a) in which mercaptoethanol, like glycerol, acts as a more efficient

acceptor for the glucosyl radical than water. The time-dependent inactivation can be most readily explained as due to the reduction of one or more disulphide bonds essential to the integrity of the enzyme. As with rhodanese (Sörbo 1953), the alternative explanation of thiohemiacetal formation from an essential carbonyl group may be excluded by the ineffectiveness of other carbonyl reagents, e.g. phenyl-hydrazine, as inhibitors (Jermyn 1955a). Combining the present results with the earlier ones on inhibition by heavy metals, it appears that both a disulphide bond or bonds and a thiol group or groups are necessary for full enzyme activity.

## V. Effects of Type 2 Activators and Inhibitors

Type 2 effects in the *S. atra* $\beta$-glucosidase were originally diagnosed (Jermyn 1955b) by the fact that plots of the reciprocal of reaction velocity (Lineweaver and Burk 1934) for the affected reactions gave straight lines parallel to those for the unaffected reaction. Later (Jermyn 1957a) reasons were given for supposing that these

Table 1

EFFECTS OF 10 MINUTES PRE-INCUBATION WITH MERCAPTOETHANOL ON THE ENZYMIC ACTIVITY OF S. ATRA $\beta$-GLUCOSIDASE

| Mercaptoethanol Concn. (M) | | Measured Enzyme Activity (arbitrary units) | Mercaptoethanol Concn. (M) | | Measured Enzyme Activity (arbitrary units) |
|---|---|---|---|---|---|
| plus Enzyme, Pre-incubate for 10 min | Incubate with Substrate and Enzyme for 20 min | | plus Enzyme, Pre-incubate for 10 min | Incubate with Substrate and Enzyme for 20 min | |
| 5·0 | 1·0 | 139 | 0 | 1·0 | 639 |
| 1·0 | 0·2 | 630 | 0 | 0·2 | 695 |
| 0·2 | 0·04 | 415 | 0 | 0·04 | 443 |
| 0·04 | 0·008 | 376 | 0 | 0·008 | 395 |

effects were due to interactions of the active agents with the acceptor centre of the enzyme. If type 2 activators act by attaching themselves to the acceptor centre and act as more efficient acceptors than water, then some of the glucose which would have been liberated in the non-activated reaction should, in the presence of activator, now appear as a $\beta$-glucoside of the activating molecule. Type 2 inhibitors, which merely block the acceptor centre, should leave the ratio liberated glucose/liberated aglucone unchanged. This point is readily checked by following the liberation of both *p*-nitrophenyl and glucose from the enzymic hydrolysis of *p*-nitrophenyl $\beta$-glucoside in both the presence and absence of the inhibitors or activators. Glycerol (non-reducing and showing pure type 2 behaviour) was an obvious choice as an activator but the choice of the non-reducing phenyl $\alpha$-cellobioside as the inhibitor was attended by some disadvantages, since its solubility did not allow it to be used at a concentration show-ing profound depression of activity. The results of these experiments are set out in Figures 3 and 4. These accord with the hypothesis, but it was obviously desirable to

confirm the postulated mechanism further by the direct isloation of a glyceryl glucoside from the enzymic digest. The following procedure was therefore adopted.

*p*-Nitrophenyl β-glucoside (500 mg), *S. atra* β-glucosidase (50 units in concentrated solution), glycerol (3·5 ml), and McIlvaine buffer (10 ml, pH 5·0) were made up to 50 ml and the whole incubated for 24 hr at 28°C. The product was deionized by passage through a column of "Bio-Deminrolit" (more than 99 per cent. of the *p*-nitrophenol removed) and the effluent concentrated to small volume. The concentrate was absorbed on a carbon–cellulose column and eluted by an ethanol gradient. A first carbohydrate peak which was eluted by water alone and gave tests for reducing sugar was obviously glucose. This was followed by a second peak of non-reducing carbohydrate at 7·5 per cent. ethanol (using the "small column" with 100 per cent. ethanol feed under the conditions already described in detail in Jermyn 1957c). The fractions containing this material were evaporated *in vacuo* but the residue (190 mg of syrup, $[\alpha]_D^{20} - 26\cdot2°$ (*c*, 5 in $H_2O$)) could not be induced to crystallize. A sample was dried at 80°C under high vacuum and submitted to the C.S.I.R.O. Microanalytical Laboratory. (Found: C, 42·1; H, 7·2; O, 50·2 %. Calc. for $C_9H_{18}O_8$: C, 42·5; H, 7·1; O, 50·4%.) Periodate oxidation according to Jackson (1944) gave an $HIO_4$ uptake of 2·94 moles per mole of $C_9H_{18}O_8$; 1-glyceryl-β-D-glucoside contains three glycol groups per mol.

1-Glyceryl-β-D-glucoside has been synthesized biochemically by the action of emulsin on glycerol plus glucose (Bourquelot, Bridel, and Aubry 1915; $[\alpha]_D^{20} - 27\cdot25°$) and chemically (Karrer and Hurwitz 1922; $[\alpha]_D^{18} - 27\cdot7°$), in both cases as a noncrystalline syrup. The optical activity is no guide to purity since a fresh asymmetric centre is created at the 2-position of glycerol and the two isomers will be formed in unknown proportions. Indeed, Bourquelot *et al.* give good reasons for supposing that their product was a mixture of two substances. In the circumstances the properties of the present product agree as well as can be expected with those already reported, and it is equally probably a mixture.

Like all alkyl β-glucosides the 1-glyceryl compound is highly resistant to the *S. atra* β-glucosidase and the glyceryl glucoside resulting from carrying out the hydrolysis under the conditions indicated in Figure 4 itself remained unhydrolysed in the presence of active enzyme for an indefinite period. It may therefore be concluded that there is convincing evidence of the transfer of a glucosyl residue to glycerol but not to phenyl α-cellobioside. An interesting point illustrated by Figure 3 is that there is virtually no transfer of the glucose residue to the product of hydrolysis (glucose) so that the amounts of liberated glucose and aglucone coincide at any time. This is in marked contrast to the behaviour of the *Aspergillus oryzae* β-glucosidase (Jermyn and Thomas 1953) and agrees with the fact that glucose does not show type 2 effects except at very high concentrations (Jermyn 1955b).

## VI. INHIBITORS OF β-GLUCOSIDASE

### (a) Threose and Erythrose

An interesting test of a hypothesis about competitive inhibitors of the *S. atra* β-glucosidase can be made by using sugars that cannot assume the pyranose ring form. The simplest cases that can be considered are those of the two tetroses, D-threose and

D-erythrose, which cannot exist in a ring form larger than the five-membered furanose ring.

It has been postulated (Jermyn 1955b) that the essential configuration for a molecule to act as a competitive inhibitor of the glucosidase is given by the portion of the D-glucose molecule namely:

$$
\begin{array}{c}
\text{OH} \\
| \\
\text{H}\!-\!\overset{}{\underset{6}{\text{C}}}\!-\!\text{H} \\
| \\
\overset{5}{\underset{\substack{\text{H}\\\text{OH}}}{\text{C}}} \\
| \\
\underset{3}{\text{C}} \\
| \\
\text{H}
\end{array}
$$
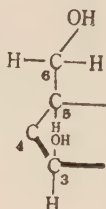
and thus D-xylose can have the configuration of a competitive inhibitor in neither its
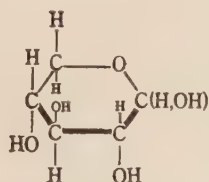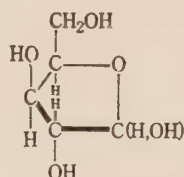


D–Xylose (pyranose)    D–Xylose (furanose)

pyranose nor furanose forms and is not, in fact, a competitive inhibitor. We may write the furanose and straight-chain forms of D-threose and D-erythrose as shown and
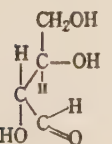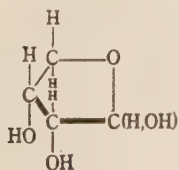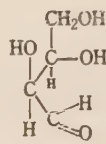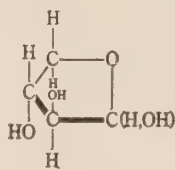


D–Erythrose                D–Threose

make the further prediction that none of these molecules will be effective competitive inhibitors. The actual experimental results are set forth in Figure 5.

Both sugars appear to be pure type 2 activators and, indeed, D-erythrose is the most powerful of these yet investigated. There is no trace of activity as competitive inhibitors at the concentrations tested.

## (b) Deoxy Sugars

It has been found (Jermyn 1955b) that both D-glucose and D-mannose are competitive inhibitors of the *S. atra* β-glucosidase and hence surmised that the steric configuration about carbon atom 2 ($C_2$) of the D-glucopyranose ring is not very important in deciding affinity for the substrate centre of the enzyme. 2-Deoxy-D-glucose, which has no hydroxyl group on $C_2$ supplies an interesting test of this hypothesis. Figure 6 demonstrates that this sugar is indeed a pure competitive inhibitor with $K_i = 126 \times 10^{-5}$M at both concentrations. A comparison with D-glucose ($K_i = 19 \times 10^{-5}$M) and D-mannose ($43 \times 10^{-5}$M) shows that both the steric configuration and the addenda at $C_2$ have an effect on the affinity for the substrate centre. Some significance that cannot at present be assessed must also be attached to the fact that the absence of a hydroxyl group at $C_2$ seems to abolish all affinity for the acceptor centre.
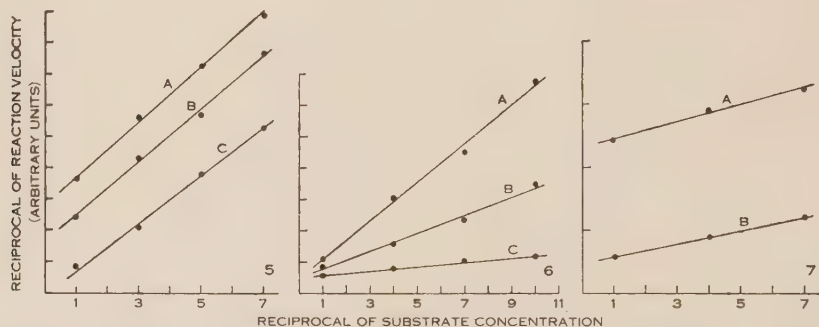


Fig. 5.—Effect of D-erythrose and D-threose on the enzymic hydrolysis of *p*-nitrophenyl β-D-glucoside by the *S. atra* β-glucosidase under the standard conditions: *A*, no addition; *B*, D-threose, $2.5 \times 10^{-2}$M; *C*, D-erythrose, $7 \times 10^{-3}$M. Unit substrate concentration, $10^{-3}$M.

Fig. 6.—Effect of 2-deoxy-D-glucose on the enzymic hydrolysis of *p*-nitrophenyl β-D-glucoside by the *S. atra* β-glucosidase under the standard conditions: *A*, no addition; *B*, $4 \times 10^{-3}$M; *C*, $10^{-2}$M. Unit substrate concentration, $10^{-3}$M.

Fig. 7.—Effect of D-rhamnose on the enzymic hydrolysis of *p*-nitrophenyl β-D-glucoside by the *S. atra* β-glucosidase under the standard conditions: *A*, no addition; *B*, D-rhamnose, $10^{-1}$M. Unit substrate concentration, $10^{-3}$M.

It was surmised at the same time that the polarity of the substituent on $C_6$, rather than the nature of this substituent or the configuration about $C_5$, was essential for effectiveness as a competitive inhibitor. This hypothesis can be partly checked by testing the behaviour of D-rhamnose (D-mannose with the hydroxyl group on $C_6$ removed). Figure 7 shows that D-rhamnose is a pure type 2 inhibitor and that there appears to be no affinity for the substrate centre   Replacement of the –$CH_2OH$ substituent on $C_5$ by both H (D-xylose) and $CH_3$ (D-rhamnose) appears to abolish this affinity.

## (c) Monomethylated Glucoses

Some idea of the nature of the interaction between D-glucose and the substrate centre of the enzyme can be gained by using compounds in which the hydroxyl groups of D-glucose have been replaced by methoxyl groups. The substituent remains polar but the possibility of forming hydrogen bonds through the hydroxyl group has been

destroyed. The simplest cases that can be considered are those of the six $O$-mono-methyl derivatives of glucose. A consideration of their interaction with the enzyme allows the influence of the five hydroxyls of glucose to be evaluated separately.

The six sets of experimental results in Figure 8 show the results for inhibitor concentrations selected to demonstrate particularly clearly the general effect of each
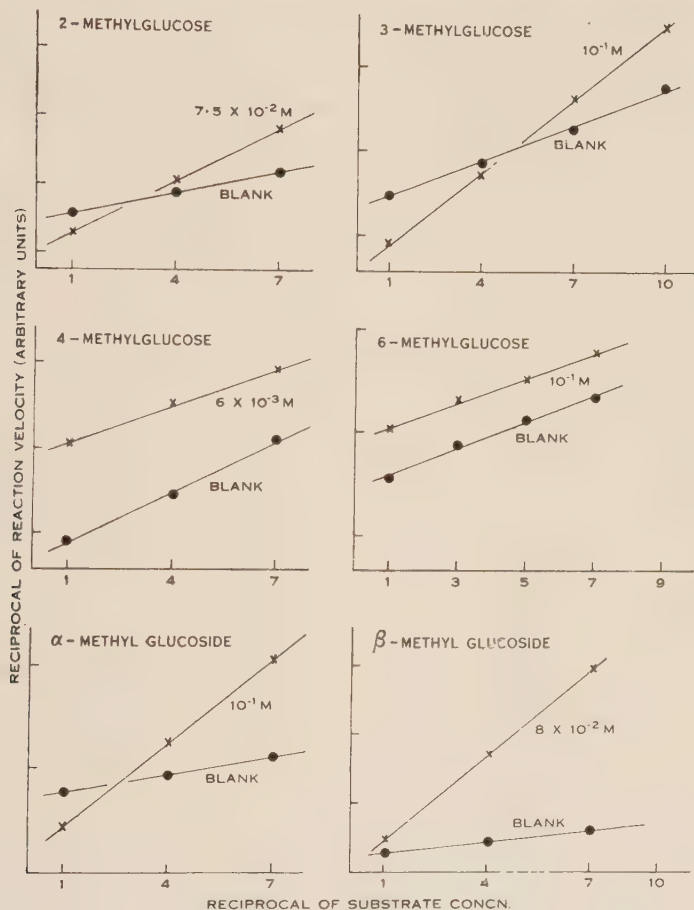


Fig. 8.—Effect of the six mono-$O$-methyl derivatives of D-glucopyranose on the enzymic hydrolysis of $p$-nitrophenyl $\beta$-D-glucoside by the *S. atra* $\beta$-glucosidase under the standard conditions. Unit substrate concentration, $10^{-3}$M throughout.

inhibitor. However, the same tendencies and comparable values of $K_i$ were observed with a number of inhibitor concentrations in each case. The observations of this and the preceding subsection are combined with some from a previous paper (Jermyn 1955$b$) in Table 2.

The results suggest the following summation of the requirements that molecules related to D-glucose should have *some* affinity for the substrate centre of the $\beta$-glucosidase of *S. atra*. Conclusions arrived at earlier (Jermyn 1955$b$) are incorporated in brackets. The nature and configurations of the groups attached to $C_1$ and

$C_2$ of D-glucopyranose (or even the existence of $C_1$ and $C_2$) are immaterial; there must be a polar but not necessarily a hydroxyl group on $C_3$ (and the configuration about $C_3$ must be that of D-glucose); there must be a hydroxyl group on $C_4$ (but the con- figuration about $C_4$ is immaterial); the grouping attached to $C_5$ must be $-C(6)H_2OH$ (but the configuration about $C_5$ is immaterial). The magnitude of the affinity (here taken as the numerical value of $K_i$) is then determined by further more detailed influences of the geometry of the molecule.

There seems at present to be no clue that will allow a general statement about whether a given molecule can be bound at the acceptor centre or whether it will act as an activator (acceptor) or inhibitor.

TABLE 2

EFFECTS OF SOME ALTERATIONS TO THE STRUCTURE OF THE D-GLUCOSE MOLECULE ON EFFECTIVE- NESS AS AN INHIBITOR OF S. ATRA $\beta$-GLUCOSIDASE

| Substance | Alteration to the D-Glucose Molecule | $K_i$ as Competitive Inhibitor ($\times 10^{-5}$M) | Type 2 Effects |
|---|---|---|---|
| D-Glucose | | 19 | Slight inhibitor |
| D-Gluconolactone | $= O$ for $H + OH$ on $C_1$ | 0·32 | |
| Methyl a-D-glucoside | $-OCH_3$ for $-OH$ on $C_1$ | 2700 | Powerful activator |
| Methyl $\beta$-D-glucoside | $-OCH_3$ for $-OH$ on $C_1$ | 1200 | Slight activator |
| Aryl $\beta$-D-glucosides | $-OAr$ for $-OH$ on $C_1$ | 2–600 | Some are slight inhibitors |
| D-Mannose | Inversion of $-OH$ on $C_2$ | 43 | None |
| 2-Deoxy-D-glucose | $-H$ for $-OH$ on $C_2$ | 126 | None |
| 2-O-Methyl-D-glucose | $-OCH_3$ for $-OH$ on $C_2$ | 4000 | Activator |
| 3-O-Methyl-D-glucose | $-OCH_3$ for $-OH$ on $C_3$ | 9000 | Activator |
| D-Galactose | Inversion of $-OH$ on $C_4$ | 1000 | Very slight inhibitor |
| 4-O-Methyl-D-glucose | $-OCH_3$ for $-OH$ on $C_4$ | Non-competitive | Powerful inhibitor |
| 6-O-Methyl-D-glucose | $-OCH_3$ for $-OH$ on $C_6$ | Non-competitive | Inhibitor |
| D-Rhamnose | $-H$ for $-OH$ on $C_6$ | Non-competitive | Inhibitor |

## VII. ENZYMATIC HYDROLYSIS OF SUBSTITUTED $p$-NITROPHENYL $\beta$-GLUCOSIDES

Various elements of the structure of an aryl $\beta$-glucoside will allow a molecule to be bound at the substrate centre of the *S. atra* $\beta$-glucosidase, but it earlier appeared that the complete structure was necessary for this molecule to be a substrate (Jermyn 1955b). The use of monosubstituted $p$-nitrophenyl $\beta$-glucosides appears to be a way of checking this in more detail.

Certain 6-substituted glucosides are readily prepared but the solubility of $p$-nitrophenyl 6-benzoyl-$\beta$-glucoside in water was too low ($< 10^{-4}$M at 28°C) and that of the 6-$p$-toluensulphonyl derivative ($5 \times 10^{-4}$M at 28°C) barely high enough for any results to be obtained. The results with the latter compound are included in Table 3 but it should be realized that the forced use of low concentrations of substrate, together with the very high enzyme concentrations necessary to demonstrate very low activities may make these uncertain with in an order of magnitude. The other four compounds recorded are the four monomethyl ethers of $p$-nitrophenyl-$\beta$-D-gluco- pyranoside. The negative results for the 2- and 4-substituted compounds held for

the highest enzyme and substrate concentrations that could be obtained and it is certain that in both cases the rate of enzymic hydrolysis at 28°C is less than $10^{-6}$ of that of the unsubstituted glucoside. For the 3-substituted compounds all that can be stated is that there is positive evidence of enzymic hydrolysis but that the combined effects of low solubility, limited stability, and small available quantities of the substrate make it impossible to assign accurate numerical values as yet.

Before the significance of the results in Table 3 can be assessed two further questions must be answered. Are the 6-substituted derivatives actually hydrolysed by the same enzyme as the unsubstituted glucoside? Are the 2- and 4-methyl derivatives bound to the enzyme and not hydrolysed or are they not bound at all? The first question can be answered by finding out whether the enzymic hydrolysis of the 6-methyl derivative is competitively inhibited by the same substances, with the same $K_i$ values, as that of the unsubstituted glucoside. The second by examining whether the 2- and 4-methyl derivatives will themselves act as competitive inhibitors.

TABLE 3

SUBSTITUTED $p$-NITROPHENYL $\beta$-D-GLUCOSIDES AS SUBSTRATES FOR $\beta$-GLUCOSIDASE
OF S. ATRA

| Substituent | Behaviour | $K_m$ ($\times 10^{-5}$M) | $\dfrac{k_3 \text{ (unsubstituted)}}{k_3 \text{ (substituted)}}$ |
|---|---|---|---|
| 2-O-Methyl- | Non-substrate | —— | —— |
| 3-O-Methyl- | Substrate | c. 10,000 | Too high for accurate measurement. Relative reaction rate c. 380 at $10^{-3}$M |
| 4-O-Methyl- | Non-substrate | —— | —— |
| 6-O-Methyl- | Substrate | 360 | 3·4 |
| 6-O-Tosyl- | Substrate | 29 | 130 |
| None | Substrate | 5 | 1 |

It was found that both phenyl $\beta$-D-glucoside and D-glucose were competitive inhibitors of the enzymic hydrolysis of $p$-nitrophenyl 6-O-methyl-$\beta$-D-glucoside. When it is remembered (Jermyn 1955b) that it was impossible to make $K_m$ and $K_i$ agree more than approximately for a large number of aryl $\beta$-glucosides tested with the S. atra enzyme it is apparent that the results set out in Table 4 agree as nearly as can be expected with the hypothesis that the unsubstituted and 6-methyl glucosides are being hydrolysed by the same enzyme. The opposite trends in the value of $K_i$ with increasing phenyl $\beta$-glucoside concentration seems to be linked with the experimental fact that the deviation from Michaelis-Menten kinetics is negative at high substrate concentration (i.e. excess substrate inhibits) for $p$-nitrophenyl $\beta$-glucoside and positive (excess substrate activates) for the 6-methyl derivative.

The concentrations of the 2- and 4-methyl derivatives used as inhibitors could not be raised above $5 \times 10^{-3}$M because of difficulties with excessively large blanks and the fact that the 4-methyl derivative is readily salted out of solution. However, these concentrations were enough to show that the 2-methyl derivative is certainly not

bound and the 4-methyl compound only weakly bound ($K_i = c. 300 \times 10^{-5}$M) at the acceptor centre (Fig. 9) and that these compounds are to be classed as type 2 inhibitors with practically identical affinities for the acceptor centre (identical $V_{max.}$ in Fig. 9). The conclusion therefore follows that no prediction can be made about the behaviour of the substituted glucoside from that of the substituted sugar. Table 5 shows that, in fact, the behaviour of the two groups tends to be opposite.

TABLE 4

COMPETITIVE INHIBITION OF S. ATRA $\beta$-GLUCOSIDASE BY D-GLUCOSE AND PHENYL $\beta$-D-GLUCOSIDE

| Substrate | $K_i$ ($\times 10^{-5}$M) for D-Glucose at Indicated Concentration as Inhibitor | | | $K_i$ ($\times 10^{-5}$M) for Phenyl $\beta$-D-glucoside at Indicated Concentration as Inhibitor | | |
|---|---|---|---|---|---|---|
| | $10^{-4}$M | $3 \times 10^{-4}$M | $10^{-3}$M | $10^{-4}$M | $3 \times 10^{-4}$M | $10^{-3}$M |
| p-Nitrophenyl 6-O-methyl-$\beta$-D-glucoside | 21 | 43 | 60 | 32 | 34 | 33 |
| p-Nitrophenyl $\beta$-D-glucoside | 27 | 21 | 17 | 21 | 19 | 20 |

Since both the 4-methyl and 6-methyl compounds have about the same affinity for the substrate centre, the conclusion may be drawn that 4-methyl substitution in p-nitrophenyl $\beta$-glucoside produces a molecule that will not act as a substrate. If the

TABLE 5

INTERACTION OF METHYL-SUBSTITUTED D-GLUCOSES AND p-NITROPHENYL $\beta$-D-GLUCOSIDES WITH THE SUBSTRATE AND ACCEPTOR CENTRES OF S. ATRA $\beta$-GLUCOSIDASE

| | 2-O-Methyl- | | 3-O-Methyl- | | 4-O-Methyl- | | 6-O-Methyl- | |
|---|---|---|---|---|---|---|---|---|
| | Sugar | Glucoside | Sugar | Glucoside | Sugar | Glucoside | Sugar | Glucoside |
| Substrate centre | Blocked | No interaction | Blocked | Substrate | No interaction | Blocked | No interaction | Substrate |
| Acceptor centre | Activated | Blocked | Activated | ? | Blocked | Blocked | Blocked | Activated (?) |

binding is primarily due to the $\beta$-glucosidic linkage, then perhaps 4-methyl substitution blocks some vital attachment necessary for enzymic action, since the same substittution abolishes the affinity of D-glucose for the substrate centre.

There may well be some correlation between the non-interaction of p-nitrophenyl 2-O-methyl-$\beta$-D-glucoside with the substrate centre and the fact that this

2-methyl substitution virtually makes it impossible for electrons to be withdrawn from the C(1)—O bond as demonstrated by the extreme resistance of this substance to alkaline hydrolysis. This matter will be discussed in detail elsewhere.

## VIII. General Discussion

The general principles on which the experimental results here presented have been interpreted have been given by Gottschalk (1950) and discussed elsewhere by Jermyn (1957a) but there seem to be some general points that are worth emphasizing. Consideration of the ideas of Dodgson, Spencer, and Williams (1956) and Ebersole, Gutentag, and Wilson (1944) showed the author that the interpretation placed on the existence of "type 2" activation and inhibition (see Part VI of this series (Jermyn
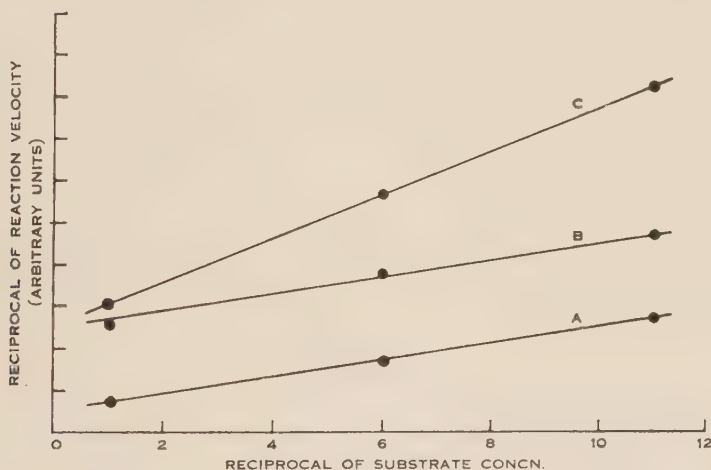


Fig. 9.—Inhibition by $p$-nitrophenyl 4-$O$-methyl- and 2-$O$-methyl-$\beta$-D-glucosides of the enzymic hydrolysis of $p$-nitrophenyl $\beta$-D-glucoside by the *S. atra* $\beta$-glucosidase under the standard conditions: $A$, $5 \times 10^{-3}$M $p$-nitrophenyl 4-$O$-methyl-$\beta$-D-glucoside; $B$, $5 \times 10^{-3}$M $p$-nitrophenyl 2-$O$-methyl-$\beta$-D-glucoside; $C$, no addition. Unit substrate concn., $10^{-3}$M.

1955b)) was too narrow. The conclusion drawn there that these kinetic effects depended on combination at an "acceptor" centre was correct, but there was no need for the special relationship between $K_i$, $k_2$, and $k_3$ that had been deduced. Abandonment of this special relationship makes it possible to explain rationally a great deal of the kinetics of the *S. atra* $\beta$-glucosidase and, by implication, of many other carbohydrases, as due to the existence and independent specifications of a pair of active centres—the "substrate" and "acceptor" centres.

Examples of the typical Lineweaver-Burk plots given by what the author had called type 2 inhibition and Dodgson *et al.* call "anticompetitive" inhibition can be found scattered throughout the biochemical literature. One case is quoted by Jermyn (1955b); Dodgson *et al.* quote several others and show how the interpretation to be placed on this phenomenon has generally been overlooked. Levvy in a series of papers has shown that there are two active centres in $\beta$-glucuronidase and that one of them

can be "blocked" by excess substrate (Levvy and Karunairatnam 1951). The interpretation of "competitive" and anticompetitive inhibition as due to the blocking of substrate and acceptor centres thus provides an attractive explanation of a wide range of experimental facts. None the less, the exact interpretation to be placed on the existence of these two centres is extremely uncertain. Thus D-glucose reacts primarily with the substrate centre as an inhibitor and secondarily also as an inhibitor with the acceptor centre, and $p$-nitrophenyl $\beta$-D-glucoside primarily with the substrate centre as a substrate and secondarily with the acceptor centre as an inhibitor; on the other hand, 6-$O$-methyl-D-glucose reacts only with the acceptor centre as an inhibitor but $p$-nitrophenyl 6-$O$-methyl-$\beta$-D-glucoside primarily with the substrate centre as a substrate and secondarily with the acceptor centre as an activator. Facts like these suggest that the substrate and acceptor centres may be only two aspects of the same site but even the "double displacement" mechanism of Koshland (1953) which seems to be supported by the kinetics of the *S. atra* $\beta$-glucosidase (Jermyn 1957a) also seems to involve two neighbouring non-identical sites. It must be admitted that no fully meaningful picture of the mechanisms involved can be put forward at the present time. Nor can the reaction of complex sugar derivatives with the acceptor site be harmonized in a simple theory with that of simple molecules such as water, glycerol, and mercaptoethanol.

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# THE EFFECT OF AUXINS ON THE BINDING OF PECTIN METHYL-ESTERASE TO CELL WALLS

By K. T. Glasziou* and Sue D. Inglis*

*Summary*

Further studies on the binding of pectin methylesterase (PME) to cell wall preparations are described. The PME of extracts of wall preparations from artichoke tubers was separated into three fractions, A, B, and C. Two similar fractions (A and C) were obtained from tobacco pith wall preparations. The amount of fraction A type PME which could be adsorbed to wall preparations was increased by the addition of 2,4-dichlorophenoxyacetic acid (2,4-D), but not by calcium ions. Neither 2,4-D nor calcium increased the adsorption of fraction B, but calcium and not 2,4-D increased the amount of fraction C adsorbed to the wall preparations.

Exhaustive extraction of intact tobacco pith disks with dilute salt solutions also gave two PME fractions, an extracted one probably identical with A, and another which was not extracted. The amount of PME in any extraction reflected the partitioning of this fraction between the disks and the solution. The equilibrium could be shifted in either direction by the addition of 2,4-D at physiological concentrations.

The results are consistent with the earlier hypothesis that the extension properties of primary cell walls may be controlled by the auxin-induced adsorption of PME to cell wall sites.

A manometric method for the assay of PME is described.

## I. Introduction

Previously it was shown (Glasziou 1957a, 1957b) that the auxins 3-indolyl-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and α-naphthaleneacetic acid (NAA) were effective in binding pectin methylesterase (PME) to cell wall preparations from which the enzyme had been split with strong salt solutions. Each auxin had an optimum activity at about $10^{-10}$M. Two other types of binding were observed, one taking place in the absence of added cofactors (non-specific adsorption) and another requiring calcium ions.

It was postulated that the auxin-controlled binding of PME could control the extension properites of the primary cell walls by regulating the amount of PME adsorbed. Immobilization would reduce the activity of the enzyme, and favour an increased degree of methylation of the pectic components of the wall. In a preliminary report (Glasziou 1958) details were given of experiments in which whole tobacco pith disks were placed in salt solutions at pH 7·0. Under these conditions PME was partitioned between the disks and the salt solution and the equilibrium position could be altered with 2,4-D. For this reaction the optimum auxin activity was in the range $10^{-5}$–$10^{-6}$M. The results were interpreted as showing that auxin controlled the binding of PME to sites in the disks in a similar manner to the *in vitro* adsorption of PME to cell wall preparations.

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Recently, Tagawa and Bonner (1957) have examined the effects of magnesium, calcium, potassium, and auxin on the elastic and plastic properties of the *Avena* coleoptile. Their experiments have given strong support to the hypothesis that the primary change which results in increased cell wall extensibility is a methylation towards pectin. Evidence that auxin can affect the rate of incorporation of methyl groups into pectin has been provided by Ordin, Cleland, and Bonner (1957) working with methyl-labelled methionine.

This paper reports further details of the reactions by which PME is adsorbed to cell wall preparations from Jerusalem artichoke and tobacco pith and also describes some aspects of the relationship between auxin and PME in whole tobacco pith disks.

## II. Materials and Methods

### (a) Artichoke Tubers

The tubers used in all the experiments described had been stored in damp sand at 4–6°C for from 1 to 10 weeks.

### (b) Tobacco Pith

Pith was obtained from autumn-sown plants of *Nicotiana tabacum* L. These plants grew slowly over the winter period and had reached a height of 2–3 ft when harvested in the spring. Pith was removed with a cork borer from along the whole length of the stem except for the tip. Where required, disks about 1 mm in thickness were cut, pooled, and aerated for 2–3 days in distilled water (which was changed at frequent intervals).

### (c) Enzyme Assays

The pH titrimetric method described previously (Glasziou 1957b) was used with the minor modification that the pH was kept as constant as possible at 7·0. In addition, a manometric method was developed for assays which required higher sensitivity. This method used the standard procedures of Warburg manometry for the measurement of acid production by the evolution of $CO_2$ from a bicarbonate buffer. The reaction mixture consisted of 4·0 ml 1 per cent. (w/v) citrus pectin in 0·1M NaCl adjusted to pH 7·5 and 0·5 ml 0·23M $NaHCO_3$ in the main compartment, and 0·5 ml enzyme solution in the side-arm. The gas phase was 95 per cent. $N_2$ and 5 per cent. $CO_2$. The volume of the manometer vessels used was c. 25 c.c. Linearity was obtained at a reaction rate of 15 $\mu l$ $CO_2$/min over an initial period of 10 min. In general, it was found advisable to use smaller amounts of enzyme than would give this rate. The reaction mixture was varied for special purposes and satisfactory results were achieved using 2 per cent. pectin.

### (d) PME Units

For the purposes of this work, a PME unit was defined as that amount of enzyme which at pH 7·0 and 20°C gave a rate of acid production equivalent to 1·0 ml 0·005N acid in 10 min. The assay solution consisted of 10 ml of 0·5 per cent. (w/v) citrus pectin in 0·1M NaCl.

## III. Results

### (a) Non-specific and IAA-induced Adsorption of PME to a Wall Preparation from Artichoke Tuber which had been Stored in the Cold

It was previously found (Glasziou 1957b) that storing artichoke tubers in the cold resulted in the disappearance of the auxin-induced binding of PME and the appearance of increased non-specific adsorption. In experiments with the same batch of artichoke tubers Adamson and Adamson (1957) found that actively growing
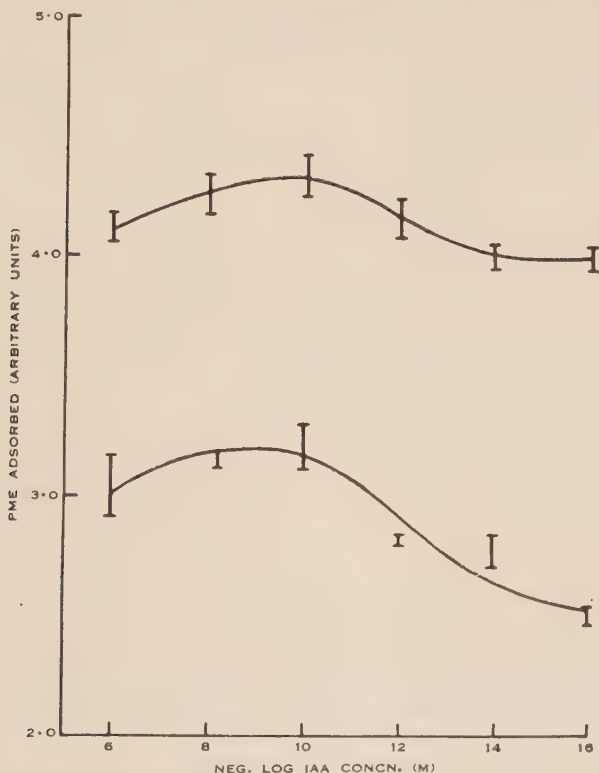


Fig. 1.—The effect of IAA on the adsorption of PME to wall preparations from cold-stored artichoke tubers. The tuber tissue was pretreated in an IAA medium (5 mg/l), and harvested during the phase of IAA-induced expansion (40 hr). Curves shown are for two separate experiments. Vertical lines represent twice the standard errors of the means. Values at $1 \times 10^{-10}$M IAA are significantly different from values at $10^{-14}$ and $10^{-16}$M IAA for the upper curve and $10^{-16}$M IAA for the lower curve ($P < 0.04$).

tubers had no lag period for the effect of auxins on tissue expansion, but that after cold storage there was a lag before expansion commenced. No extensive studies have been made of these effects. However, the following results are recorded as they may have an important bearing on the lag period for auxin response found in many storage or mature tissues.

Artichoke tubers which had been stored for 2 months at 4°C were cut into sections about 1 mm in thickness, washed for several hours in running tap-water,

then incubated with aeration for 3 days in an IAA-containing solution (5 mg/l).  At this stage, the IAA-treated tissue had expanded approximately 30 per cent. more than controls in water.  The IAA-treated tissue was then homogenized and PME and a wall fraction prepared as previously described (Glasziou 1957b).

In two experiments in which the tissue had been taken through the lag period by IAA pretreatment, an auxin-induced binding of PME to the wall fraction was observed, although high non-specific adsorption was also present (Fig. 1).  As many previous experiments with stored artichoke tubers had failed to show any marked effect of auxins on the binding of PME, it appears likely that auxin pretreatment of the tissue had induced formation of the system for the auxin-controlled binding of PME to the wall sites.
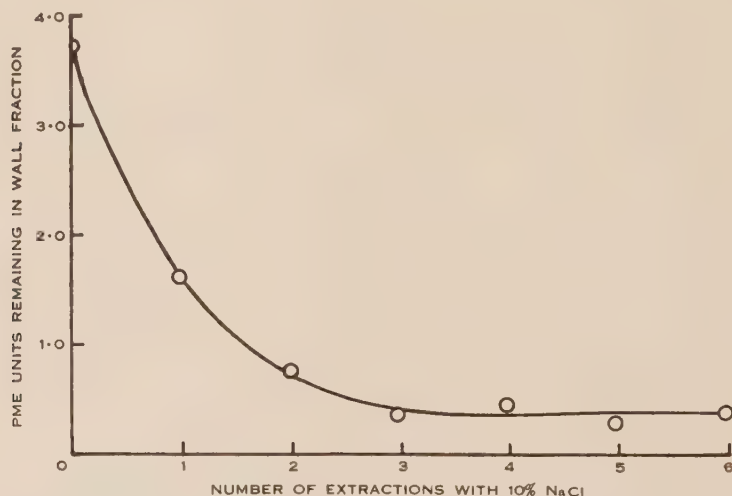


Fig. 2.—The residual PME activity of a wall fraction prepared from artichoke tubers.  Equal portions of a wall fraction were extracted one to to six times with 10 ml portions of 10 per cent. NaCl, then filtered, and the PME activity of the residue determined.  Boiling of the wall fraction destroyed the activity.

(b) *The Heterogeneity of PME and PME Adsorption Sites in Artichoke and Tobacco Tissues*

Evidence has accumulated which indicates that PME may be heterogeneous.  For example, McColloch, Moyer, and Kertesz (1946) purified tomato PME by precipitating the enzyme by the use of 10 per cent. NaCl solution.  On the first precipitation 60 per cent. of the original activity was lost, but no further losses were sustained by repeated precipitations.  Kertesz (1936) found that PME from tobacco was almost quantitatively adsorbed on decolorizing charcoal but Mehlitz (1932) found that PME from alfalfa was not adsorbed.

Experiments involving cell fractionation and purification of the PME of artichoke tubers and tobacco pith have indicated that the enzyme from these tissues is heterogeneous.  For convenience the enzymes have been classified into several types based on their adsorption characteristics.

The first type of PME was bound to wall preparations and could not be removed by treatment with 10 per cent. NaCl at pH 7·0, though this treatment removed most of the PME associated with the walls. To demonstrate this point, equal amounts of a wall preparation were extracted one to six times with 10 per cent. NaCl. In Figure 2 the PME activity of the extracted wall residue is plotted against the number of extractions made. No more enzyme was removed by extracting more than three times and the walls retained a small amount of PME activity. The residual activity was destroyed by heat treatment at 100°C for 10 min.

TABLE 1

THE FRACTIONATION OF PME ELUTED FROM WALL PREPARATIONS OF JERUSALEM ARTICHOKE AND TOBACCO PITH

The tissues were homogenized, centrifuged four times at 500 $g$, the PME extracted with 10 per cent. NaCl, and the extract dialysed overnight (see text for fractionation procedure)

| Description of Fraction | Total PME Units in Fraction | | | | |
| | Artichoke Tubers | | | Tobacco Pith | |
| | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 1 | Expt. 2 |
|---|---|---|---|---|---|
| Dialysate | 109 | 100 | 150 | 100 | 79 |
| Filtrate after supercel treatment of dialysate (fraction A) | 31 | 37 | 38 | 5·5 | 4·5 |
| Supercel extracted with 0·1M NaCl (fraction B) | 57 | 31 | 87 | 3·0 | — |
| Above supercel extracted with 1·0M NaCl (fraction C) | 33 | 14 | 8 | 53·5 | 48 |

The fractionation of the PME contained in dialysates of 10 per cent. NaCl extracts from wall preparations was carried out with "Hyflo" supercel. When treated in this way, part of the PME activity was adsorbed to the supercel, and a part remained in solution. The soluble enzyme was called fraction A. The enzymes which were adsorbed on the supercel have been called fraction B or C according to whether they were eluted with 0·1 or 1·0M NaCl. Table 1 shows the division of the PME activity between the three fractions in five separate experiments with wall preparations from artichoke tuber or tobacco pith. Results given in Table 2 show that about five extractions of the supercel with 0·1M NaCl sufficed to remove all of the fraction B enzyme, but that further extraction with 1·0M NaCl resulted in a new fraction (fraction C) being brought into solution.

When each of the fractions A, B, and C were treated a second time with "Hyflo" supercel, most of the activity of fraction A and fraction C type PME was recovered in the same fraction in which they had originally appeared. With fraction B much of the activity was lost but a portion of the activity was recovered as fraction C (Table 3). This portion may have been due to contamination of fraction B with fraction C enzyme.

Many experiments have been carried out in attempting to demonstrate recombination of PME with the wall fraction in the presence of 2,4-D or calcium ions,

TABLE 2

THE ELUTION OF FRACTION B AND FRACTION C TYPE PME OF ARTICHOKE TISSUES FROM "HYFLO" SUPERCEL

The tissues were homogenized, centrifuged three times at 500 $g$, the PME extracted with 10 per cent. NaCl, and the extract dialysed overnight. The dialysate was treated with "Hyflo" supercel (3 g/100 ml), and filtered after 15 min. The residue was extracted firstly with successive 15-ml portions of 0·1M NaCl (to give fraction B) and then with 1·0M NaCl (to give fraction C)

| Fraction | Eluate | Total PME Units Eluted at each Treatment | Fraction | Eluate | Total PME Units Eluted at each Treatment |
|---|---|---|---|---|---|
| B | 1 | 30 | C | 1 | 23 |
|   | 2 | 16 |   | 2 | 8 |
|   | 3 | 7 |   | 3 | 2 |
|   | 4 | 3 |   | 4 | 0 |
|   | 5 | 1 |   |   |   |
|   | 6 | 0 |   |   |   |

TABLE 3

THE EFFECT OF A SECOND TREATMENT WITH "HYFLO" SUPERCEL ON FRACTION A, B, AND C TYPE PME FROM ARTICHOKE TUBERS

Fractions B and C were dialysed before treatment to remove NaCl. The volumes of all fractions were adjusted to give approximately the same enzyme concentration as in the initial dialysate. Each fraction was treated with supercel as before (see text) and after filtration elutions were made firstly with 0·1M NaCl then with 1·0M NaCl

| Solution Assayed | Percentage of Original Activity Recovered | | | | |
|---|---|---|---|---|---|
|  | Fraction A | | Fraction B | | Fraction C |
|  | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 | Expt. 1 |
| Filtrate after supercel treatment | 78 | 76 | 10† | 7† | 15† |
| Eluate of supercel with 0·1M NaCl | 8 | * | 1 | 3 | 0 |
| Eluate of supercel with 1·0M NaCl | 3 | * | 24 | 29 | 74 |

*Not determined.        †These figures uncertain because of dilution factor.

added at concentrations which had been shown to give marked effects in other preparations (Glasziou 1957a, 1957b). An increase in the amount of PME adsorbed

to the wall fraction from artichokes in the presence of 2,4-D has been demonstrated for fraction A and a slight effect for fraction B which was probably due to contamination of fraction A type enzyme (Table 4).  Non-specific adsorption occurred with both fraction A and fraction B, but no calcium effect has been demonstrated for either.  A marked calcium effect was observed for fraction C obtained from tobacco pith.

Further evidence for the heterogeneity of PME or PME binding is described in Sections III(c) and III(d) for experiments on whole tobacco pith sections.

TABLE 4

THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) ON THE ADSORPTION OF FRACTION A AND B TYPE PME TO A WALL PREPARATION OF ARTICHOKE TUBERS

The complete system contained 2 g wall fraction, 2·44 PME units of fraction A or 6·15 PME units of fraction B, and 2,4-D at a final concentration of $1 \times 10^{-9}$M. For experiments with fraction A the final volume was 25 ml and for fraction B 10 ml.  After incubation for 90 min at 19°C, the wall residues were filtered, washed with 100 ml 0·001M phosphate buffer pH 7·0 (in 10-ml portions), and the PME activity of the residues determined

| Type of PME Fraction | System Components | Units of PME Adsorbed to Wall | |
|---|---|---|---|
| | | Expt. 1 | Expt. 2 |
| A | No 2,4-D | 0·52 | 0·57 |
| | Complete | 1·07 | 1·07 |
| B | No 2,4-D | 1·40 | 1·33 |
| | Complete | 1·57 | 1·58 |

Experiments carried out with fractions A, B, and C obtained from an artichoke wall preparation indicated that none of these fractions would hydrolyse methyl butyrate or ethyl acetate, further indicating that none have general esterase activity. No extensive experiments have yet been carried out to determine specificity, pH optima, cation requirments, etc.

### (c) The PME Activity of Whole Tobacco Pith Sections

Bryan and Newcomb (1954) grew tobacco pith disks on agar–sucrose medium containing IAA.  A large increase in fresh weight of the disks occurred compared with the control (no IAA) and the cells enlarged and tended to separate.  When whole disks were assayed for PME, considerable activity was measured.  Control disks, the cells of which had not separated from each other, had about 30 per cent. of the activity of an equivalent tissue homogenate, while IAA-treated disks with greater exposure of the cell surfaces to the medium had 60 per cent. of the activity of an equivalent homogenate.  It was concluded that the high activity of intact cells towards the non-penetrating pectin molecules provided good evidence for the surface localization of the enzyme.

These experiments have been repeated in part and the high PME activity of intact disks confirmed. However, in view of evidence for the heterogeneity of PME, it seemed that Bryan and Newcomb's (1954) observations could be interpreted in a different way, since the effect of IAA may have been to increase the proportion of one type of PME (i.e. a surface-localized enzyme). It was thought that vacuum infiltration of whole disks with a PME assay solution containing pectin and salt should increase the initial rate of hydrolysis of the pectin, provided that the pectin penetrated the intercellular spaces and the enzyme was truly localized in the cell surfaces. When this was done the rate of hydrolysis of pectin increased in both the control and the

TABLE 5

THE HYDROLYSIS OF METHYL ESTER GROUPS OF PECTIN BY WHOLE DISKS OF TOBACCO PITH

The complete system contained 2 g fresh weight tobacco pith disks and 10 ml 0·5 per cent. citrus pectin containing 0·1M NaCl. The PME activity was determined by measuring the steady rate of pectin hydrolysis at pH 7·0

| System | Steady Rate of Pectin Hydrolysis (ml 0·005N acid produced in 10 min) |
|---|---|
| Complete | 1·20 |
| Vacuum infiltrated* | 1·20 |
| Plus 0·15M $Ca^{++}$ | 1·51 |
| No NaCl† | — |

*The time to reach the steady rate (about 20 min) was not decreased by vacuum infiltration.

†PME requires cations for optimum activity. Removal of the disks after 20 min and addition of NaCl to 0·1M showed that no PME was extracted.

infiltrated disks over an initial period of about 20 min. A steady rate of hydrolysis then ensued and there was no difference in the steady rate between the treatments. As vacuum infiltration had not decreased the time for reaching the steady rate, the effect of removing the disks was examined. It was then found that in each case more than 90 per cent. of the PME activity of the whole disks remained behind in the assay solution.

PME is normally assayed in the presence of cations (see MacDonnell, Jansen, and Lineweaver 1945), and, whereas Bryan and Newcomb (1954) used calcium in the assay medium, in these experiments sodium was used. Calcium was therefore added to the assay medium at a final concentration of 0·15M, and was found to increase substantially the amount of PME extracted from the disks. Table 5 gives results for the effects of vacuum infiltration, removal of cations, and addition of calcium on the steady rate of pectin hydrolysis for tobacco pith disks.

Investigation showed that the PME activity measured in whole tobacco pith disks was due almost entirely to the extraction of the enzyme by the salt of the assay medium.   As the assays were made by determing the rate of acid production, it was

TABLE 6

THE BASAL RATE OF ACID PRODUCTION BY DISKS OF TOBACCO PITH IN THE ABSENCE OF PECTIN

The complete system contained 1 g fresh weight tobacco pith disks in 10 ml water containing NaCl and 2,4-D at the concentration shown.   The pH was adjusted continually to 7·0 by the addition of 0·005N NaOH

| System | Rate of Acid Production (ml 0·005N acid/10 min) |
|---|---|
| No NaCl | 0·062 |
| No NaCl; $5 \times 10^{-6}$M 2,4-D | 0·055 |
| 0·1M NaCl | 0·13 |
| 0·1M NaCl; $5 \times 10^{-6}$M 2,4-D | 0·10 |
| 0·2M NaCl | 0·13 |
| 0·2M NaCl; $5 \times 10^{-6}$M 2,4-D | 0·11 |

necessary to determine the basal rate of acid production by the disks in the absence of pectin, and with varying salt concentration.   Results for one such experiment are given in Table 6 and show that the rate was low enough to be neglected in most experiments.   The effect of 2,4-D is also shown.

TABLE 7

THE EXTRACTION OF PME FROM DISKS OF TOBACCO PITH WITH SODIUM CHLORIDE

Disks (1 g fresh weight) were extracted with 0·5 per cent. citrus pectin (12 ml) containing 0·16M NaCl and at pH 7·0 until a steady rate of pectin hydrolysis was reached.   The disks were then transferred to a fresh assay solution and the extraction repeated.   After five extractions, the disks were homogenized, and the PME activity of the brei determined

| | Extraction | | | | | |
|---|---|---|---|---|---|---|
| System measured: | 1st | 2nd | 3rd | 4th | 5th | Brei of disks after 5th extraction |
| PME units extracted: | 0·58 | 0·28 | 0·14 | 0·07 | 0·00 | 2·10 |

To gain knowledge of the mechanism of extraction of PME from the whole disks with salt solutions, disks were extracted in pectin and NaCl until a steady rate of acid production was reached, then transferred to a fresh solution, and again assayed until the steady rate was reached.   The process was continued until no more PME could be extracted from the disks, which were then homogenized and the activity of brei determined.   The results given in Table 7 show that for that particular batch of disks the amount of PME which could be extracted in this way was about

30 per cent. of the total enzyme originally present. This result is further evidence for the heterogeneity of PME and PME adsorption sites in plant tissue. The results also indicate that the steady rate of pectin hydrolysis represents an equilibrium position in which the enzyme is partitioned between the salt solution and the disks. PME adsorbed to the disks did not contribute to the steady rate of hydrolysis of pectin (there was no significant difference for the rate of pectin hydrolysis when the disks were removed from the assay solution after the first extraction, provided the basal rate of acid production of the disks was considered).
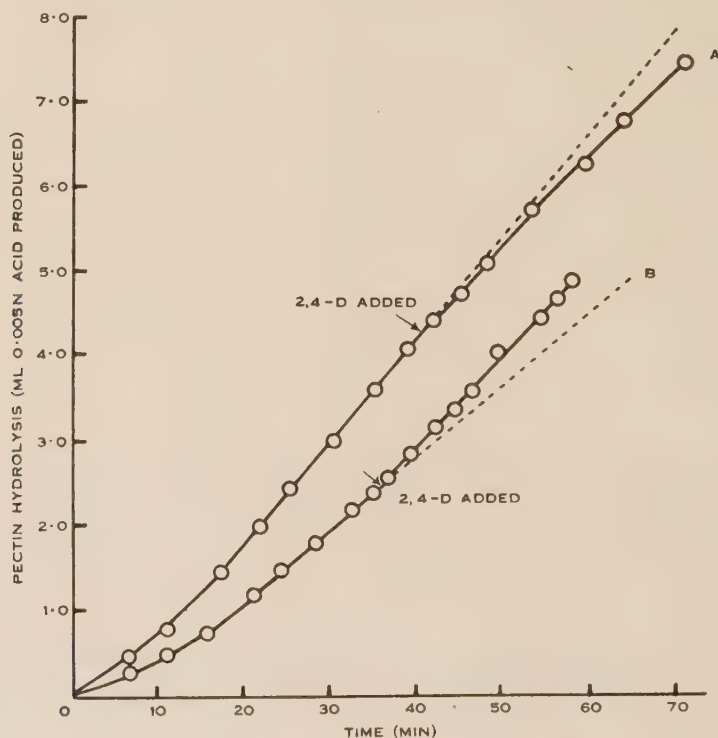


Fig. 3.—The effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on the extraction of PME from tobacco pith disks. The 2,4-D was added at a final concentration of $1 \times 10^{-5}$M when a steady rate of pectin hydrolysis had been attained. The shift in rate on addition of 2,4-D was due to a shift in the equilibrium position for partitioning of PME between solution and disks. Curves A and B are for disks from different plants.

### (d) The Effect of 2,4-D on the Extraction of PME from Tobacco Pith Disks

Figure 3 shows the effect of adding 2,4-D to tobacco pith disks which had reached a steady rate of pectin hydrolysis before addition of auxin. For the two sets of results given, disks from different plants were used. The disks shown in curve B had been pretreated for 1 hr in 0·2M NaCl and then transferred to the assay solution. These results show that, depending on the tissue and conditions, the equilibrium position for partitioning of PME between the assay solution and the disks can be shifted in either direction. Results published previously (Glasziou 1958) have shown that

activity of the extracted enzyme is not affected by 2,4-D and that the optimal concentration of 2,4-D for increasing the amount of PME retained by the disks was in the range of $10^{-5}$–$10^{-6}$M.

When PME extracted from whole disks with a salt solution at pH 7·0 was fractionated with "Hyflo" supercel in the manner previously described the activity was lost, apparently through denaturation.

The salt concentration necessary to extract PME from whole disks varied when the pith was obtained from different tobacco plants. Usually 0·1M NaCl sufficed but higher concentrations were sometimes required. Divalent cations were more effective than monovalent. For example, in one lot of disks very little enzyme was extracted with 0·15M NaCl but an appreciable amount was extracted with the same concentration of $CaCl_2$.
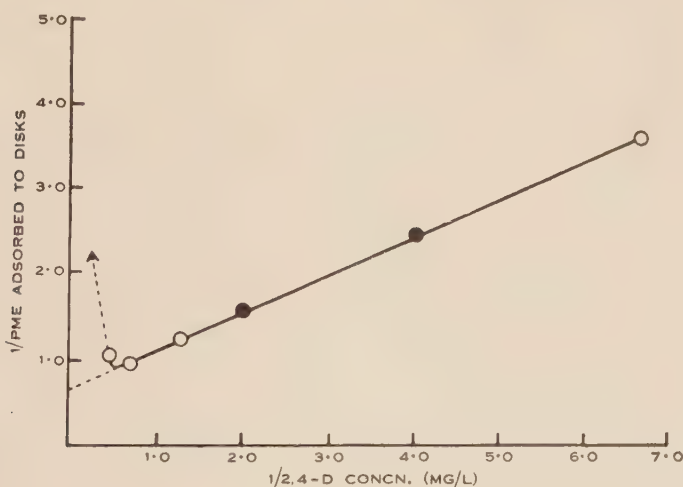


Fig. 4.—Double reciprocal plot of data from Figure 2(a) of Glasziou (1958). The PME adsorbed to disks due to the addition of 2,4-D was obtained by subtraction from the amount adsorbed at zero concn. ○ Points from experimental data; ● points from smooth curve.

Several techniques were used for measuring the effect of 2,4-D on the extraction of PME from disks. The method which yielded the most accurate results was to place the disks in an assay solution and record the steady rate of acid production attained after about 20 min by measuring the amount of alkali required to keep the pH at 7·0. Each assay took about 1 hr and required careful attention to keep the pH constant between limits of 0·2 of a pH unit. A second technique was to incubate the tissue in salt solution and to determine the PME activity of an aliquot of the solution after removal of the disks. To do this it was necessary to buffer the salt solutions, as the disks tend to lower the pH which results in marked changes in the amount of enzyme extracted. The addition of buffer decreased the accuracy of the enzyme assays as it decreased the sharpness of the titration end-point. A third method was to assay the enzyme by using the manometric technique. The disadvantages of this method were threefold. There was firstly the inherent error of Warburg

manometry (about 5 per cent.). Secondly, the addition of bicarbonate and subsequent gassing with $CO_2$ gave a period of about 15 min in which there was inadequate pH control. Because the method was much more sensitive than the pH titration, the amount of tissue had to be restricted to keep the gas changes within reasonable limits and this increased the sampling error.

Although the general effect of 2,4-D has been established using the three techniques cited, the technical difficulties and the variability of the degree of response to auxin between pith obtained from different plants has prevented us from making extensive kinetic studies on the effect of varying 2,4-D concentrations. However, it is worth recording that the double reciprocal plot of 1/PME adsorbed against 1/auxin concentration (Fig. 4) calculated from published data (Fig. 2($a$) of Glasziou 1958) is of the form previously noted for auxin-controlled PME adsorption to isolated wall fractions.

## IV. Discussion

Three types of adsorption of PME to cell wall preparations have been observed. First, there was an auxin-controlled mechanism in which the amount of enzyme adsorbed was increased by increasing concentrations of auxin to an optimum above which a decrease occurred. A second type of adsorption occurred in the absence of added cofactors and may be similar to that postulated by Lineweaver and Ballou (1945) to explain the inihibition of PME from alfalfa by pectic acid. Alfalfa PME was considered to be positively charged below pH 8·5, and to form salt linkages with the dissociated carboxyl groups of pectic acid at lower pH values. An increase of pH, or the addition of cations, was considered to break these bonds and release the enzyme from inhibition. If this postulate is correct, non-specific adsorption of PME may depend on the amount and degree of esterification of protopectin in the wall preparations, and also on the pH and salt concentration. Further work will be carried out on this problem.

Thirdly, as has been reported previously (Glasziou 1957$b$), calcium is highly effective in promoting the binding of PME to wall preparations from artichoke tubers: but, at similar concentration, calcium will increase the amount of PME extracted from whole tobacco pith disks. Of the three PME fractions obtained after treatment with "Hyflo" supercel, only fraction C has been observed to give increased recombination with wall preparations in the presence of calcium.

Though negative results are of doubtful value in experiments in which PME was recombined with wall preparations in the presence or absence of added cofactors, the following interpretation is given. Besides the residual PME activity of the cell wall preparations, there were two types of PME in tobacco pith tissue and three types in the artichoke tuber tissue. On fractionation of crude PME extracts using "Hyflo" supercel, the enzyme which was affected by auxins appeared in fraction A in extracts from artichoke wall preparations but was mostly denatured in extracts from tobacco wall preparations. The difference may have been due to protective substances (protein etc.) in the artichoke extracts. The auxin-controlled enzyme, which could be extracted from whole tobacco pith disks, was also denatured by treatment with "Hyflo" supercel. The fraction C type PME was present in both artichoke and

tobacco tissues. It showed no non-specific adsorption and no auxin effects but could be recombined with some wall preparations provided calcium ions were present at a concentration of about $10^{-2}$M. The fraction B type PME from artichoke tubers was not affected by auxins but showed non-specific adsorption to wall preparations.

Bryan and Newcomb (1954) observed that IAA-induced enlargement of tobacco pith disks was accompanied by an increase in PME activity to almost twice the value for control sections after 120 hr. They also measured the PME activity of whole tobacco pith disks in which IAA-induced cell expansion had occurred and showed a greater percentage of total activity of an equivalent homogenate than for controls in which cell enlargement had not taken place. The results were interpreted as evidence for the surface localization of PME. Our results showed that the PME activity of whole tobacco pith disks, measured in a pectin–salt assay mixture, was a measure of the amount of enzyme extracted from the disks. Therefore, Bryan and Newcomb's results may have been due to formation of a greater proportion of the type of PME which can be extracted by salt solutions, and which is under auxin control. The enzyme is present in tobacco pith cells (probably in the walls) and is extracted by salt solutions. The amount extracted depends on the pH, type and concentration of cation, and auxin concentration. The equilibrium position for partitioning the enzyme between the solution and the disks depends on the balance between the factors mentioned. The soluble enzyme is not inhibited by auxins; the amount extracted from disks is increased by calcium. The effects of pH and salt concentration indicate that this enzyme may bind to pectate in the walls of the disks in a similar manner to that described by Lineweaver and Ballou (1945) for the binding of alfalfa PME to pectate *in vitro*.

Previously, it was reported (Glasziou 1957a) that the *in vitro* inhibition of PME by IAA described by Bryan and Newcomb could not be repeated. Subsequent work has confirmed that auxins have no *in vitro* effect on the activity of PME extracted from tobacco pith disks, or PME associated with or extracted from wall preparations. However, the anomaly may be due to a difference in interpretation of the term *in vitro*. Reference to Table 3 of Bryan and Newcomb indicates that the experiments may have been carried out on whole pith disks, in which case the results are analogous to those reported here. A plot of their data shows that the curve obtained for IAA is very similar to that found for 2,4-D (Glasziou 1958, Fig. 2(b)) over the low concentration range.

The results reported in this paper are consistent with the previous hypothesis that auxins control extension growth in plants by controlling the adsorption of PME to sites within the cell wall (the greater the adsorption, the smaller the PME activity). Increased adsorption would permit increased methylation of the pectic substances and enhanced plastic properties (i.e. reduced wall pressure).

The role of the other types of PME in cellular metabolism in not clear. These enzymes, like a number of other hydrolytic enzymes, may be more or less inactive in the intact cell. Alternatively, the various pectin-hydrolysing enzymes may be specific for different chain lengths of the polygalacturonides as in the fatty acid-activating enzymes. Methylesterase enzymes of different specificities would provide a possible explanation of the results of Ordin, Cleland, and Bonner (1957)

showing that auxin affected the rate of incorporation of labelled methyl groups into pectin or their rate of loss from pectin, but did not affect incorporation or loss from protopectin.

Many of the far-reaching effects of auxins may be explicable in terms of the drain on the methyl group and other metabolic pools in the cell when the activity of PME is altered, and in certain types of cell these secondary effects may well be more important than the primary effect on the wall properties.

Some of the possible metabolic interrelationships concerned in determining the properties of the primary cell wall are summarized below:

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# EFFECTS OF SOME INORGANIC NITROGENOUS SUBSTANCES ON GROWTH AND NITROGEN ASSIMILATION OF YOUNG PLANT EMBRYOS *IN VITRO*

By A. H. G. C. Rijven*

### Summary

Young excised embryos of *Anagallis arvensis* L. (Primulaceae) and of the cruciferous species *Arabidopsis thaliana* (L.) Heynh., *Capsella bursa-pastoris* (L.) Moench., and *Sisymbrium orientale* L. were fed with inorganic and organic nitrogenous compounds whilst they were kept in "sitting drop" cultures for periods of 48 and 96 hr.

The growth response to nitrate was slight or absent in all species. The inability to utilize nitrate could not be overcome by adding ascorbic acid to the medium. Also embryos did not show adaptation to nitrate in experiments in which nutritional conditions for the induction of necessary enzymes were appropriate.

Direct tests showed nitrate reductase to be inducible in immature embryos of *Capsella* and wheat.

In contrast with nitrate, striking growth responses to nitrite (0·1–4·0 mM) were found in the cruciferous species but not in *Anagallis*.

With respect to ammonium salts there was also a difference between the species: *Anagallis* showed a marked response, but in the cruciferous species the response was slight and formed a curious contrast with the response to nitrite. The effect of urea followed the ammonium pattern.

All species responded vigorously to L-alanine, L-glutamic acid, and L-glutamine.

Effects of nitrogenous compounds on "insoluble nitrogen" content per embryo were similar to those on increase in length.

## I. Introduction

Since the introduction of coconut milk as a nutrient for the culture of young excised plant embryos (van Overbeek, Conklin, and Blakeslee 1941), the evidence has suggested that the amino acids are largely responsible for the beneficial effect of this and other natural nutrient sources (Sanders and Burkholder 1948). It has been shown that the growth of embryos of various species is stimulated considerably by the addition of a number of single amino acids and amides to a basal synthetic medium irrespective of whether it contains nitrate or not (Haagen Smit, Siu, and Wilson 1945; Rijven 1952, 1955, 1956; Paris *et al.* 1953).

On the other hand, mature germinating embryos are apparently able to utilize nitrate nitrogen. Harris (1956) has shown that, during the first week of incubation in darkness, oat embryos isolated from soaked grains grow on nitrate about as well as on the best amino acid mixture tested. He also found that, in the presence of nitrate, addition of amino acids failed to stimulate growth.

These facts indicate that there is a contrast in the nitrogen nutrition of the plant during its embryonic development and after germination. The specific question, however, whether young plant embryos are able to utilize inorganic nitrogen, above

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all nitrate, has not been answered satisfactorily. It would reveal an interesting aspect of the developmental physiology of the plant if it were established that development from the pre- into the post-germinal type of growth coincided with the activation or first appearance of the system responsible for the assimilation of inorganic nitrogen.

As a contribution towards this problem the author undertook a series of feeding experiments with young excised embryos using the "sitting drop" culture technique. Growth was measured as increase in length, but, as a check on conclusions based on these measurements, nitrogen determinations were carried out on cultured embryos.

The main aim in the following feeding experiments was to determine whether steps of the assimilatory pathway operating in higher plants are deficient in embryos. Though the number of species studied was small, it appeared that profound differences exist between species in their ability to utilize various nitrogenous compounds.

## II. Material and Methods

"Torpedo-shaped" embryos of the following species were used:

*Anagallis arvensis* L., red variety

*Arabidopsis thaliana* (L.) Heynh., a genetically pure line originally from Estonia

*Capsella bursa-pastoris* (L.) Moench.

*Sisymbrium orientale* L.

The procedure for culturing the embryos has already been described (Rijven 1952) and involves a rather short period (2 and 4 days) test of increase in length of embryos excised from ovules and explanted in a modified "sitting drop" culture technique. The culture cells contain up to 20 embryos which are suspended in separate drops of about $30\mu l$ of liquid medium between two glass slides; this permits the measurement of their lengths by means of a microscope fitted with an eyepiece micrometer. Handling of the drops and of the embryos is done with sterilized braking-pipettes.

The basal medium was made up by adding 120 g sucrose to 1 l. salt solution consisting of:

| | | | |
|---|---|---|---|
| $KH_2PO_4$ | 2·0 mM | $MnCl_2$ | 0·02 µM |
| $K_2HPO_4$ | 0·5 mM | $H_3BO_3$ | 0·01 µM |
| $CaCl_2$ | 0·5 mM | $ZnSO_4$ | 0·01 µM |
| $MgSO_4$ | 0·5 mM | $Na_2MoO_4$ | 0·002 µM |
| Fe (chelated | | $CuSO_4$ | 0·001 µM |
| with EDTA*) | 5·0 mg/l | | |

*(handwritten corrections in the right-hand column: 2.0 above $MnCl_2$; 1.0 above $H_3BO_3$; 1.0 above $ZnSO_4$)*

Inorganic substances used were of A.R. grade and the amino acids were products of L. Light & Co. Ltd., England. The media, with pH $6·0 \pm 0·2$, were sterilized by filtration through sintered-glass filter funnels. Embryos were dissected and washed in the basal medium, the cultures were kept at 25°C in a dark incubator.

As the initial lengths of excised embryos vary, a considerable amount of calculation was involved in the evaluation of the observations. It was assumed

*Ethylenediaminetetra-acetic acid.

that the regression of length increment on initial length was a linear one, i.e.

$$y = a + bx,$$

where $y$ = length increment, and $x$ = initial length. It was possible to pool the regression slopes ($b$) on the different treatments in a given experiment. Then an analysis of covariance was made and for the different treatments the adjusted mean of the length increments at the mean initial length of all observations calculated. These are the more prominent figures given in the tables of Section III. Conservative estimates of the least significant difference at the 1 per cent. level are included in the tables. These estimates are conservative in so far as they are based on the two treatments of the experiment which happen to have the lowest numbers of degrees of freedom. They therefore safeguard against an overestimation of the level of significance in any difference found within a column of a table.

For the determination of insoluble nitrogen the technique of Levy (cf. Glick 1949), was followed with minor deviations. In order to work in the range of nitrogen quantities for which the method is designed, i.e. $0.5$–$10\,\mu g$, at least five embryos had to be used per sample. The embryos were first fixed in a mixture of 40 per cent. formaldehyde, 5 parts, acetic acid, 5 parts, 70 per cent. ethanol, 90 parts, and kept in this mixture for days or even weeks. They were washed with ethanol and transferred to the digestion tubes (1 by 7 cm). The ethanol was evaporated. To each tube $0.1$ ml of Levy's digestion mixture was added and digestion was carried out whilst the tubes were seated in 1-cm deep closely fitting cavities in an iron block on a hot plate. After digestion and cooling, $1.4$ ml distilled water was added and then, with vigorous aeration, $0.6$ ml Nessler reagent (after Folin and Wu). The absorption was measured at 420 m$\mu$. Blanks and standards were carried through the same procedure.

As the determination had to be carried out on samples of about five embryos and the number of replicated samples per treatment was low, it was impracticable to calculate regressions on initial length as was done for the growth measurements. However, mean values for insoluble nitrogen per embryo were adequate for the purpose in hand.

The nitrate reductase assays were carried out on a crude grindate of excised embryos prepared by crushing a suitable number of embryos with a glass rod in a small test tube prior to the addition of other reagents. The grindate was not a cell-free extract, but consisted of unbroken as well as broken cells. For each assay other additions were: $0.1$M potassium phosphate, pH $7.5$, containing 1 mM reduced glutathione ($0.2$ ml); $0.1$M $KNO_3$ ($0.1$ ml); 2 mM diphosphopyridine nucleotide ($0.5$ ml); $0.75$ mg/ml crystalline yeast alcohol dehydrogenase ($0.05$ ml); boiled pig heart extract ($0.02$ ml), prepared according to Nason and Evans (1953); water ($0.03$ ml). Incubation was for 1 hr at 30°C. The nitrite produced was determined as described by Evans and Nason (1953). The amount of nitrite in reagents and grindate prior to incubation was determined on a duplicate set of tubes and the values for the incubated tubes corrected accordingly.

Qualitative tests of the nitrate reductase activity of *Capsella* embryos were carried out on a microscale using a final volume of 150 $\mu l$. It was not practicable to obtain quantitative data on the production of nitrite under these conditions.

## III. Results

Firstly, an introductory survey of the effects on growth of a number of substances in a number of species is presented. This survey is followed by more detailed information on the effects of particular substances.

TABLE 1

EFFECTS OF SOME NITROGENOUS COMPOUNDS ON THE GROWTH OF EMBRYOS OF FOUR SPECIES

Corrected estimates of length increments of embryos incubated for 48 hr. The values are expressed as percentage of the value obtained for the control treatment and correspond within a given species to the same initial length, which is the mean of all initial lengths observed in the experiment. The statistical treatment used in this and following tables is explained in Section II of the text

| Treatment | | Length Increment as Percentage of Control | | | |
|---|---|---|---|---|---|
| Substance Added | Concn. (mM) | *Anagallis arvensis* $(100 = 150{\cdot}5\,\mu)$ | *Capsella bursa-pastoris* $(100 = 333{\cdot}2\,\mu)$ | *Arabidopsis thaliana* $(100 = 190{\cdot}1\,\mu)$ | *Sisymbrium orientale* $(100 = 295{\cdot}3\,\mu)$ |
| Nil | 0 | 100·0 | 100·0 | 100·0 | 100·0 |
| Sodium nitrate | 2 | 103·1 | 103·5 | 100·6 | 96·8 |
| Sodium nitrite | 2 | 98·3 | 201·4 | 191·6 | 264·6 |
| Ammonium sulphate | 1 | 205·9 | 114·0 | 113·5 | 103·4 |
| Urea | 1 | 238·9 | 106·7 | 123·7† | |
| L-Alanine | 2 | 339·0* | 163·0 | 157·4* | |
| L-Glutamic acid | 2 | 168·5* | | 143·8* | |
| L-Glutamine | 1 | 341·2* | | 163·7* | |
| Conservative estimate of least significant difference at 1 per cent. level | | 22·5 | 13·8 | 9·8 | 18·9 |
| Mean initial length ($\mu$) | | 550·1 | 444·5 | 269·5 | 409·1 |

*These values are derived from another experiment and the statistics given do not strictly apply to them.

†Urea concentration = 2 mM.

### (a) Introductory Survey

In Table 1 growth responses to the main nitrogenous substances used in this study are given for embryos of four species. The substances (sodium nitrate, sodium nitrite, ammonium sulphate, urea, L-alanine, L-glutamic acid, and L-glutamine) were added in nitrogen-equivalent amounts.

From this survey it is apparent that not all species show the same response pattern. Nevertheless, the absence of a significant stimulative response to sodium nitrate as contrasted with the consistent response to amino acids in all cases is especially noteworthy. It is also seen that the three cruciferous species behave essentially alike and, as a group, show conspicuous differences from *Anagallis*. These differences are found in the responses to ammonium sulphate and urea, and to sodium

nitrite. In *Anagallis* the effects of ammonium sulphate and urea are considerable, but in the cruciferous species effects are absent or relatively inconspicuous. On the other hand, no response to sodium nitrite is shown by *Anagallis*, whilst the response to the same substance in the cruciferous species stands out amongst the other compounds tested.

### (b) Nitrate

Though no significant effects by nitrate were observed in the above experiments, significant effects were noted in some other experiments. Such effects, however, were always of a low order of magnitude, i.e. in the neighbourhood of 10–20 per cent. in excess of the value obtained for the control treatment.

TABLE 2

EFFECT OF DIFFERENT CONCENTRATIONS OF SODIUM NITRATE ON GROWTH
OF EMBRYOS OF ARABIDOPSIS THALIANA

Corrected estimates of length increments of embryos incubated for 48 hr.
The values correspond to an initial length of 277·0 $\mu$ which is the mean of all
initial lengths observed

| Sodium Nitrate Concn. (mM) | Length Increment ($\mu$) |
|---|---|
| Nil | 154·9 |
| 2·0 | 169·0 |
| 4·0 | 170·6 |
| 6·0 | 171·8 |
| 10·0 | 174·9 |
| Conservative estimate of least significant difference at 1 per cent. level | 12·8 |
| Pooled regression slope | 0·30 |

A limited ability to respond to nitrate was found in an experiment with *Arabidopsis* in which different concentrations were applied up to 10 mM (Table 2). For incubation periods of 48 hr effects by nitrate are not usual, but they appear regularly after 96 hr (Tables 3 and 4).

A few experiments have been made to test the possibility of ascorbic acid improving the utilization of nitrate. It has been shown by Virtanen and von Hausen (1949) that cotyledonless pea seedlings grow poorly on a medium with nitrate as nitrogen source, but considerably if ammonium sulphate is used instead. However, these seedlings grow about as vigorously on nitrate as on ammonium nitrogen when ascorbic acid is added to the nitrate-containing medium. According to these authors, ascorbic acid fulfilled the requirement for a low redox potential. Table 3 shows that neither in the presence nor in the absence of nitrate does ascorbic acid make *Arabidopsis* embryos grow better. It may be remarked *a priori* that *Arabidopsis* embryos do not seem comparable with the cotyledonless pea seedlings in so far as the former do not respond vigorously to ammonium nitrogen. On this basis *Anagallis* embryos would

lend themselves better for a comparison. *Anagallis* embryos proved to be stimulated by ascorbic acid (Table 3), but this stimulation was only slight and occurred both in the presence and in the absence of nitrate. Further, the effect in the presence of nitrate seems additive to the effect by nitrate alone and does not sum up to anything like the growth stimulation shown by ammonium sulphate (see also Table 1). It is concluded, therefore, that it is not here a matter of redox potential determining the faculty of embryos to respond to nitrate, and it seems logical to assume the absence, or nearly so, of a nitrate-reducing pathway.

TABLE 3

EFFECTS OF NITRATE AND ASCORBATE ON GROWTH OF EMBRYOS OF ARABIDOPSIS THALIANA AND
ANAGALLIS ARVENSIS

Corrected estimates of length increments of embryos incubated for 48 and 96 hr. The values correspond to the initial length which is the mean of all initial lengths observed for each species

| Calcium Nitrate Concn. (mM) | Ascorbate Concn. (mM) | Length Increment ($\mu$) | | | |
| | | *Arabidopsis thaliana* (mean initial length = 324·0 $\mu$) | | *Anagallis arvensis* (mean initial length = 512·0 $\mu$) | |
| | | 48 Hr | 96 Hr | 48 Hr | 96 Hr |
| Nil | Nil | 189·6 | 239·9 | 131·5 | 194·9 |
| 2·0 | Nil | 190·3 | 273·3 | 154·0 | 220·8 |
| Nil | 0·8 | 174·7 | 244·7 | 152·4 | 218·6 |
| 2·0 | 0·8 | 179·8 | 269·2 | 168·8 | 247·2 |
| Conservative estimate of least significant difference at 1 per cent. level | | 15·2 | 16·5 | 13·4 | 19·1 |
| Pooled regression slope | | 0·24 | 0·30 | −0·024 | −0·059 |

The nitrate reductase system has been shown to be inducible in *Neurospora* (Nason and Evans 1953) and in rice seedlings (Tang and Wu 1957). It is to be noted that molybdenum, which is an essential component of this system, is always present in the basal medium as a micronutrient. The time factor for bringing about a significant stimulation by nitrate in embryos, mentioned above, was thought to indicate an adaptive process. An experiment was therefore designed which might support this suggestion. It is based on the assumption that the synthesis of the enzymes of the nitrate-reducing pathway depends primarily on the presence of nitrate and of a suitable nitrogen source. Previous experience suggested that glutamine might be used (Rijven 1956; see also Tables 1 and 7). Embryos of *Anagallis* were first incubated for 22 hr in a medium containing either glutamine or glutamine plus nitrate. After these pretreatments the embryos were measured, washed, and allocated to media with and without nitrate but containing no glutamine. At the end of the second period, which

was prolonged to 112 hr on the basis of periodic inspections, the embryos were measured again. Table 4 shows that at the end of the experiment a slight difference

TABLE 4

NITRATE PRETREATMENT EXPERIMENT ON EMBRYOS OF ANAGALLIS ARVENSIS
Corrected estimates of length increments of embryos incubated for a pretreatment period of 22 hr in a glutamine-containing medium (1 mM) with and without addition of nitrate (2 mM) and of the same embryos grown for a second period of 112 hr in a basal medium without glutamine but with and without addition of nitrate (2 mM). The length increases given for the first and second period correspond to the mean initial length of first and second incubation period

| First Period (22 hr) with Glutamine | Length Increment ($\mu$) | Second Period (112 hr) without Glutamine | Length Increment ($\mu$) |
|---|---|---|---|
| Without nitrate | 243·5 | Without nitrate<br>With nitrate (2 mM) | 184·5<br>195·6 |
| With nitrate (2mM) | 246·0 | Without nitrate<br>With nitrate (2 mM) | 186·7<br>198·0 |
| Conservative estimate of least significant difference at 1 per cent. level | 10·5 | Conservative estimate of least significant difference at 1 per cent. level | 14·8 |
| Mean initial length | 479·0 | Mean initial length | 735·1 |
| Pooled regression slope | 0·116 | Pooled regression slope | −0·094 |

approaching the 1 per cent. level of significance, was found between those embryos that had not had nitrate in either the first and second period and those that had had

TABLE 5

INDUCTION OF NITRATE REDUCTASE IN IMMATURE WHEAT EMBRYOS
Wheat embryos, variety Insignia, were excised 16 days after anthesis, when the insoluble nitrogen content was 7·5 $\mu$g per embryo. Some of these were assayed immediately, others after incubation for 24 hr on sterile solutions of sucrose (120 g/l) with or without potassium nitrate (3 mM) at 25°C

| Treatment between Excision and Assay | Nitrate Reductase Activity (m$\mu$moles nitrite produced/10 embryos/hr) |
|---|---|
| None | 0·4 |
| Incubation without nitrate | 0·4 |
| Incubation with nitrate | 40·0 |

nitrate during both periods. Pretreatment with nitrate, however, did not increase the response of the embryos to nitrate in the second period. There was thus no

evidence for the presence of an adaptive mechanism from this experiment. However, direct tests have shown nitrate reductase to be inducible in *Capsella* embryos. These tests were made on a microscale and were merely qualitative. No nitrate reductase activity was found in grindates of freshly excised torpedo-shaped embryos, but there was activity in grindates of embryos which had been incubated with nitrate for 20 hr before assay.
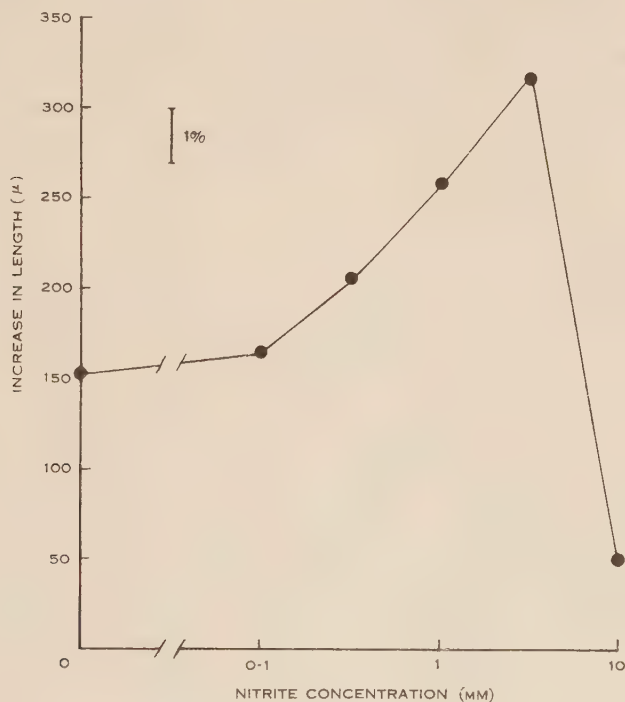


Fig. 1.—*Arabidopsis thaliana*. Effect of sodium nitrite concentration on length increment of embryos incubated for 48 hr. Mean initial length = 272·9 $\mu$. Significant difference at 1 per cent. level is indicated on the graph.

In order to obtain quantitative data on the induction of nitrate reductase, experiments were done on immature wheat embryos. The greater size of these embryos permitted the enzyme assay to be carried out on a larger scale. Table 5 shows that the grindate of freshly excised embryos had a low level of activity, and that the same was found for embryos incubated for 24 hr on a medium containing no nitrate, but that the activity increased a 100-fold when the medium contained nitrate. Apparently nitrate reductase can readily be induced in immature embryos.

### (c) *Nitrite, Hydroxylamine, and Ammonium Salts*

The marked effect of nitrite in the cruciferous species was studied further in *Arabidopsis* by applying a series of concentrations. It appeared that from 0·1 mM onward increasing stimulatory responses could be recorded but that at very high concentration (10.0 mM) nitrite is inhibitory (Fig. 1).

Hydroxylamine proved toxic or inhibitory to embryos of *Arabidopsis* in concentrations as low as 0·1 mM. Stimulative responses could not be recorded at lower concentrations.

In connection with the good responses to nitrite in this species it was considered important to check whether the low response to ammonium sulphate in the Cruciferae (Table 1) could be due to the concentration (1·0 mM) being supra-optimal. An experiment in which the concentration of ammonium sulphate ranged from 0·1 to 3·0 mM, showed that at 48 hr the best concentration (0·3 mM) gave only 14 per cent. more growth than the control. At 96 hr the best concentration (0·1 mM) gave a growth stimulation of 24 per cent. and the highest concentration was slightly inhibitive. In Section III(*e*) it will be shown that in *Capsella* a growth stimulation by ammonium sulphate is accompanied by an increase in protein content.

TABLE 6

EFFECTS OF DIFFERENT AMMONIUM ION CONCENTRATIONS IN A SUCCINIC ACID BUFFER ON THE GROWTH OF EMBRYOS OF ARABIDOPSIS THALIANA

Corrected estimates of length increments of embryos incubated for 48 and 96 hr. The values correspond with an initial length of 251·3 $\mu$ which is the mean of all initial lengths observed

| Substances Added | | | Length Increment ($\mu$) | |
|---|---|---|---|---|
| Succinic Acid (mM) | Ammonium Hydroxide (mM) | Potassium Hydroxide (mM) | 48 Hr | 96 Hr |
| Nil | Nil | Nil | 142·6 | 227·1 |
| 2 | Nil | 3·2 | 169·2 | 252·3 |
| 2 | 0·1 | 3·1 | 151·4 | 248·3 |
| 2 | 0·3 | 2·9 | 153·2 | 241·1 |
| 2 | 1·0 | 2·2 | 164·6 | 245·5 |
| 2 | 3·0 | 0·2 | 178·1 | 237·4 |
| Conservative estimate of least significant difference at 1 per cent. level | | | 12·1 | 17·2 |
| Pooled regression slope | | | 0·34 | 0·47 |

Ammonium sulphate is often credited with acidifying the medium, and such side effects could have interfered in the expression of the effect of the ammonium ion. In a search for a better system, succinic acid was chosen ($pK_1 = 4\cdot18$, $pK_2 = 5\cdot60$ at 25°C). Therefore succinic acid (2 mM) was made up to pH 5·8 with ammonium hydroxide and potassium hydroxide to give a range of ammonium ion concentrations. For this experiment the phosphate buffer concentration of the basal medium was lowered to 0·5 mM. The results (Table 6) show that at no concentration did the substitution of potassium ions with ammonium ions stimulate growth. Minor differences and trends might be due merely to the physicochemical balance between the cations affecting growth. It is, therefore, believed that the contrasting effects of nitrite and ammonium on growth are real.

### (d) Oximino Acids

At a certain stage of the investigations it was thought that nitrite nitrogen in these embryos does not follow the reductive pathway via ammonia. Wood and Hone (1948) have advocated substances like α-oximinoglutarate and α-oximinosuccinate as alternative intermediates. They showed a good response to these substances in the concentration range from 0·1 to 1·0 mM in terms of protein synthesis in oat seedlings. A series of tests, however, proved α-oximinoglutarate and α-oximinopropionate, prepared according to Wood et al. (1948), to be inhibitory to the embryos in concentrations as low as 0·1 mM, whilst at still lower concentration no stimulation could be observed.
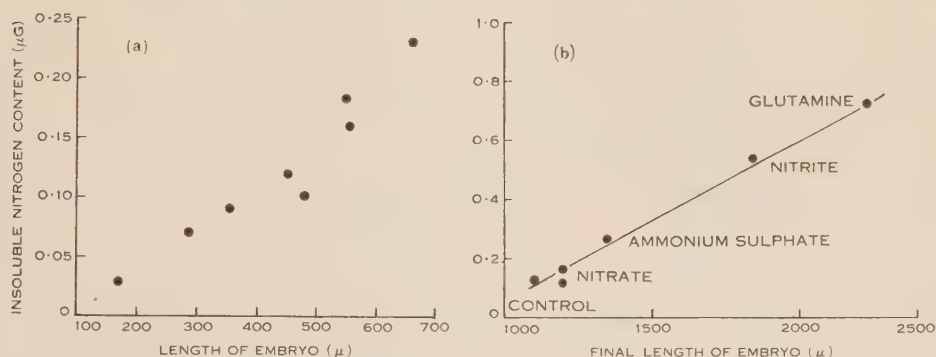


Fig. 2.—*Capsella bursa-pastoris*. (a) Relationship between insoluble nitrogen content and length, measured in alcohol, of embryos excised from ovules. The number of embryos per sample was increased to 50 for the smallest embryos. (b) Relationship between insoluble nitrogen content and length of embryos, measured in alcohol, after incubation for 96 hr with different nitrogen sources at a phosphate level of 10 mM. Data derived from Table 7.

### (e) Nitrogen Assimilation

The main reason for carrying out insoluble nitrogen determinations was to check whether there were discrepancies between effects on growth and on protein synthesis. This seemed specially necessary in face of the effects by nitrite which in the cruciferous species formed a curious contrast with effects by nitrate. Still more remarkable is the contrast with the effects of ammonium salts, for ammonia is believed to be an intermediate in nitrogen assimilation when starting from nitrite.

*Capsella* was chosen for this work because of its large embryos and Figure 2(a) gives the insoluble nitrogen content as a function of length of embryos excised from ovules.

Table 7 records data on mean final lengths and insoluble nitrogen contents of embryos incubated for 96 hr with nitrate, nitrite, ammonium sulphate, and glutamine at two levels of phosphate buffer concentration. viz. 2·5 and 10·0 mM. The higher phosphate level was tested also because it was found that the embryos grown at the usual lower phosphate level showed symptoms of germination in the nitrite and glutamine treatments. From previous experience this does not occur at the higher phosphate concentration but growth and protein synthesis appear now to be reduced by the higher concentration. The most important information, however, is thought to

TABLE 7

EFFECTS OF SOME NITROGENOUS COMPOUNDS ON NITROGEN ASSIMILATION OF EMBRYOS OF CAPSELLA BURSA-PASTORIS

Means of the final lengths of embryos, measured in alchohol, and corresponding insoluble nitrogen contents after incubation for 96 hr with different nitrogen sources at two levels of phosphate buffer concentration. The number of samples on which each value for nitrogen content is based is also given. The means of the initial lengths range from 550–600 $\mu$, corresponding with an estimated insoluble nitrogen content of 0·13–0·19 $\mu$g

| Substance Added | Phosphate Buffer (2·5 mM) | | | Phosphate Buffer (10 mM) | | | | | |
| | | | | Experiment 1 | | | Experiment 2 | | |
| | Mean Final Length ($\mu$) | Mean Final Insoluble Nitrogen Content ($\mu$g) | No. of Samples | Mean Final Length ($\mu$) | Mean Final Insoluble Nitrogen Content ($\mu$g) | No. of Samples | Mean Final Length ($\mu$) | Mean Final Insoluble Nitrogen Content ($\mu$g) | No. of Samples |
|---|---|---|---|---|---|---|---|---|---|
| Nil | 1138 | 0·22 | 4 | 1090 | 0·14 | 4 | 1190 | 0·14 | 4 |
| Sodium nitrate (2 mM) | 1200 | 0·22 | 2 | 1200 | 0·17 | 4 | | | |
| Sodium nitrite (2 mM) | 1852 | 0·74 | 3 | 1350 | 0·27 | 2 | 1846 | 0·54' | 4 |
| Ammonium sulphate (1 mM) | | | | | | | | | |
| Glutamine (2 mM) | 2883 | 1·29 | 4 | | | | 2230 | 0·74 | 4 |

be that the growth stimulation by nitrite is accompanied by a considerable increase in insoluble nitrogen content. When, irrespective of treatment by nitrogenous compound, insoluble nitrogen contents are plotted as a function of the final length of the embryos the points come close to a straight line (Fig. 2(*b*)). This demonstrates parallelism between effects on growth and protein synthesis and hence the superiority of nitrite over ammonium sulphate as a nitrogen source in this particular system.

## IV. Discussion

Amongst the results of this study, the rather inert behaviour of embryos towards nitrate as a nitrogen source stands out as of particular interest in the study of the developmental physiology of the plant.

Whilst a number of natural organic nitrogenous substances elicit considerable growth and protein synthesis in the pre-germinal phase of the plant, stimulatory effects by nitrate are established only with some difficulty using the utmost of precision available. Again, conditions which enhance nitrate utilization in germinated seedlings appeared of no avail in the embryos. In order to detect a stimulation by nitrate it is necessary to incubate the embryos over longer periods than for effects by other substances.

In looking for an explanation it seemed reasonable to assume that the embryonic state is associated with absence or low activity of the enzyme nitrate reductase. However, the direct assays for the enzyme have shown that whilst this may hold for freshly excised embryos, incubation with nitrate readily induces the enzyme. It must be concluded that the presence of the enzyme does not facilitate nitrate utilization in these embryos. In the case of the crucifers this can only be due to the enzyme not functioning because its product, nitrite, is easily utilized.

The tests on *Anagallis* (Table 1) show that notable stimulatory effects by nitrite are not general and it may be that such effects by nitrite, as noticed in the cruciferous species, are actually limited to this plant group. It has been shown already (Rijven 1956), in a survey over a wide variety of plant species, that the embryos of the Cruciferae exhibit an exceptional response pattern towards asparagine.

The main difficulty in explaining the nitrite phenomenon is to reconcile the large response to nitrite with the low responses to ammonium salts. The results of the experiments with oximino acids do not favour the hypothesis of an alternative pathway.

It is realized that these feeding experiments suffer from the drawback that such hypothetical intermediates as the oximino acids, when applied, are likely to inhibit at the higher concentrations used, but may contribute too little nitrogen for assimilation to become evident at the low concentrations in which they may be physiologically functioning. The same applies for ammonia, which indeed proved to stimulate somewhat better at lower concentrations.

Therefore, no coherent picture for the nitrogen assimilation of cruciferous embryos can as yet be offered.

The observation that, dependent on the level of the phosphate buffer, *Capsella* embryos will show symptoms of germination after 4 days of incubation in the presence

of glutamine is of interest to the general problem of maintaining the embryonic type of growth *in vitro*.

It has been assumed so far (Ziebur *et al.* 1950; Rijven 1952) that a suitably high osmotic pressure of the medium will suppress precocious germination. It should be recalled that the sugar concentration used by the author is based on the osmotic pressure in the ovule of *Capsella*, its value being isotonic with a 0·35 molal sucrose solution. This value was recently confirmed with a more reliable technique using the embryo again as an "osmometer". The fact, however, that we have not previously observed precocious germination appears now to have been due to the use of a 10 mM phosphate buffer concentration. It has now also been noticed that this high buffer concentration as compared with a 2·5 mM concentration reduces growth and protein synthesis. The primary inhibitory effect is perhaps on uptake, for Birt and Hird (1956) have noticed that phosphate inhibited uptake of amino acid in slices of carrot root.

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# RESTRICTION OF INFECTION THREADS IN NODULATION OF CLOVER AND LUCERNE

By Hilary F. Purchase*

*Summary*

Clover and lucerne roots from plants grown in tube culture were examined for infection thread formation and nodule number. The number of infection threads was about equal to the number of nodules in *Trifolium pratense* L.; this relation was shown to hold for abundantly and sparsely nodulating plants and for bacterial inoculants producing large and small numbers of nodules.

With *T. subterraneum* L., *T. repens* L., *T. incarnatum* L., and *Medicago sativa* L. infection threads were much more profuse than would be expected if each gave rise to a nodule.

Multiple infections were observed for a small proportion of nodules. Several adjacent hairs may be infected, all strands apparently contributing to formation of the one nodule; two infection strands per hair were occasionally noted.

## I. Introduction

Nutman (1956) has referred to the variety of evidence bearing on the ratio of infection thread to nodule formation. It seems that inter- and intra- cross-inoculation group compatibilities might operate either by some response between bacterium and root hair wall, or, after invasion of the root hair and cortical cells, because of failure to stimulate the "nodule primordia".

Plants inoculated in pure culture normally have their roots covered with a dense rhizosphere population (Purchase and Nutman 1957), but only a small percentage of root hairs are ever infected (Thornton 1929; McCoy 1932). Some restriction must therefore always operate at the first stage of infection. Nevertheless, in some plants this small proportion of infected root hairs much exceeds the number of nodules formed (McCoy 1932), suggesting that in these cases the second stage is more significant in determining the particular nodule complement of a root.

In the present paper the infection thread–nodule relationship has been described for several associations of red clover in tube culture; observations on other clovers and on lucerne are included for comparison.

## II. Methods

Roots taken from water culture or agar slope culture were stored in 70 per cent. alcohol. For examination they were placed on a slide, covered with a No. 0 coverslip, and the projecting hairs on either side of the root carefully examined for curling and root hair infection. Roots were stained with 10 per cent. Loeffler's alkaline methylene blue, which stains root hairs pale blue and infection threads dark blue.

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TABLE 1

COUNTS OF NODULES, INFECTED, AND CURLED ROOT HAIRS ON CLOVER ROOTS

| Species | Strain | Age (days) | Total No. of Hairs per Root | No. of Curled Hairs per Root | Curled Hairs (%) | Estimated No. of Infected Hairs per Root (I) | Infected Hairs (%) | No. of Nodules | Ratio I/N |
|---|---|---|---|---|---|---|---|---|---|
| T. pratense | SU281 | 46 | 175,000 | 9,700 | 5·6 | 0 | 0 | 1 | 0 |
| | | 60 | 124,000 | 2,100 | 1·7 | 4 | 0·003 | 2 | 2 |
| | NA30 | 46 | 21,000 | 800 | 3·8 | 4 | 0·02 | 5 | 0·8 |
| | | 60 | 86,000 | 1,200 | 1·4 | 4 | 0·01 | 16 | 0·2 |
| T. repens | SU281 | 46 | 28,000 | 300 | 1·0 | 8 | 0·03 | 19 | 0·4 |
| | | 67 | 20,000 | 200 | 1·2 | 24 | 0·12 | 19 | 1·3 |
| | NA30 | 46 | 62,000 | 1,100 | 1·8 | 72 | 0·12 | 24 | 3·0 |
| | | 67 | 59,000 | 1,800 | 3·1 | 188 | 0·32 | 7 | 26·9 |
| T. incarnatum | SU281 | 46 | 63,000 | 900 | 1·4 | 84 | 0·13 | 19 | 4·4 |
| | | 74 | 88,000 | 2,100 | 2·3 | 132 | 0·15 | 12 | 11·0 |
| | NA30 | 46 | Not counted | Not counted | — | 8 | — | 5 | 1·6 |
| | | 74 | 64,000 | 2,400 | 3·7 | 144 | 0·23 | 16· | 9·0 |
| T. subterraneum | SU281 | 46 | 123,000 | 5,300 | 4·3 | 24 | 0·02 | 15 | 1·6 |
| | | 81 | 187,000 | 9,900 | 5·3 | 724 | 0·39 | 54 | 13·4 |
| | NA30 | 46 | 144,000 | 10,800 | 7·5 | 160 | 0·11 | 18 | 8·9 |
| | | 81 | 186,000 | 28,000 | 15·0 | 976 | 0·52 | 60 | 16·3 |

In order to examine the relative numbers of curled to total root hairs, and the ratio of infection threads to nodules forming on the same roots, counting procedures were standardized as follows:

*Curled hair counts.*—Rough scale diagrams were made of the roots, which were examined with a 16-mm objective at intervals 0·5–1 cm apart by using stage co-ordinates. The field diameters varied from 1·5 to 2·1 mm according to the ocular used. The length of the roots was measured from the diagrams and estimates of the curled and total hairs were determined for the whole length of the root.

*Infected hair counts.*—For counts of infected hairs a 4-mm objective and 10 × ocular were used. High-power examination is necessary to differentiate between true infection threads and thread-like coagulation of the protoplasm which is occasionally very prevalent. Also the crossing of two hairs often gives the appearance of an infection thread under low power.

A typical root has about 10-12 rows of epidermal cells across the upper and lower surfaces of the preparation, the hairs on which could not be examined, and about 4–5 rows on either side of the root which were examined. Thus it is likely that approximately a quarter of the root hairs were available for examination and a factor of 4 was applied to the counts to give the total number of root hairs in any category.

## III. RESULTS

Certain generalizations could be made as to the distribution of root hairs along the root edge:

(1) Roots were free from root hairs in the zone 0·3–3 cm immediately behind the root tip. Older portions of the root showed a somewhat irregular distribution of root hairs, but frequently a thick mass of root hairs was observed just below the crown. The piliferous layer is not usually sloughed off under these conditions. Curling and infection of root hairs was not found to be zoned.

(2) It is necessary to examine the whole root, including laterals, to obtain reliable sampling of infected root hairs, because of their small number and irregular distribution. Curled hairs were relatively frequent and sample counts provided an estimate of their proportion.

Observations of exceptional behaviour may be mentioned:

(1) Only one case of infection in a straight root hair was noted. Single hairs on relatively isolated positions on the roots may be infected, and in a preliminary experiment one plant had only 39 root hairs, all curled and infected. Two or more infection threads may be observed on a single developing nodule. As many as nine infected hairs were once noted in 2 mm of a subterranean clover root.

(2) In one or two instances the infection thread was seen to be divided and rejoined within the root hair, giving a small loop   On several occasions two infection threads could be observed within the one root hair but it was not certain whether division of the thread had occurred from near the point of entry, as sometimes happens. They apparently resembled those described by Bieberdorf (1938) in soy-beans, and by Fahraeus (1957) in white clover.

### (a) Observation on Clover in Water Culture

The data shown in Table 1 were obtained from examination of a few roots of commercial lines of *Trifolium pratense* L. (red clover), *T. subterraneum* L. (subterranean clover); *T. repens* L. (white clover), and *T. incarnatum* L. (crimson clover) grown in seedling salts solution.

It will be seen that the number of curled root hairs per root is very variable and usually far exceeds the number of infections. Subterranean clover has a higher percentage of curled root hairs than the other species, as well as a larger total of root hairs.

In red clover the ratio of infections to nodules is about 1, but in white, crimson, and subterranean clovers the ratios are 2, 3, and 5 respectively in younger plants, and 14, 10, and 15 in older plants. These values clearly indicate the restriction in red clover as compared with the other species examined, which all show increased susceptibility to infection with age.

Uninoculated plants of all species had no infection threads and virtually no curled root hairs.

### (b) Infection in Selected Lines of T. pratense

At the suggestion of Dr. P. S. Nutman, who supplied the material, information was obtained on the relation between infection and nodulation with associations of red clover plants bearing characteristically different numbers of nodules. The plants were grown on agar slope cultures and results are shown in Table 2, which shows the counts of infected hairs per root (observed $\times 4$), together with the number of nodules per plant.

As anticipated, nodule counts increased with time to a much greater extent with the ineffective compared with the effective strain. Nodule numbers were highest with host lines selected for abundant nodulation and inoculated with the ineffective strain, and lowest with sparse selections effectively nodulated, the other two groups producing intermediate numbers of nodules. Over this wide range of nodulation capacity the ratio of nodule numbers to infected hairs remained of the same order. The number of infection threads may be expressed as a fraction or percentage of the nodules on each plant. An analysis of variance performed on the 28- and 48-day percentages after angular transformation shows no significant difference between the relationships of infection threads and nodules for the different associations.

Effective strains have more infection threads per nodule than ineffective, but this is not significant. Between different plant lines the relationship between infection threads and nodules is closely similar. It seems, therefore, that the limitation of sparse, compared with abundantly nodulating lines, or effective, compared with ineffective strains, comes into operation as early in the association as infection thread development.

With these, as with the preliminary counts, the mean ratio of infected hairs to nodules was about 1; hence it seems possible that with red clover each infection thread results in nodulation. Higher ratios (3·5–4) were noted in three out of the 26 cases cited in Table 2.

Three roots of a resistant line of red clover were examined without any infection threads being discovered.

It seems clear that for red clover the first stage of infection—the root hair entry stage—in some way defines the number of nodules formed. Whether any hairs are penetrated by bacteria which fail to form infection threads is not yet known.

TABLE 2

NUMBERS OF NODULES AND INFECTED HAIRS ON DIFFERENT ASSOCIATIONS OF RED CLOVER

| Age (days) | Abundant Clover Line | | | | Sparse Clover Line | | | |
|---|---|---|---|---|---|---|---|---|
| | Effective Strain | | Ineffective Strain | | Effective Strain | | Ineffective Strain | |
| | No. of Infected Hairs ($I$) | No. of Nodules ($N$) | No. of Infected Hairs ($I$) | No. of Nodules ($N$) | No. of Infected Hairs ($I$) | No. of Nodules ($N$) | No. of Infected Hairs ($I$) | No. of Nodules ($N$) |
| 28 | 40 | 26 | 44 | 51 | 16 | 10 | 12 | 8 |
| | 48 | 12 | 8 | 43 | 0 | 5 | 12 | 15 |
| | 16 | 21 | 48 | 53 | 8 | 8 | 4 | 7 |
| | 4 | 5 | 52 | 15 | | | 16 | 26 |
| Totals | 108 | 64 | 152 | 162 | 24 | 23 | 44 | 56 |
| Ratio $I/N$ | 1·69 | | 0·94 | | 1·04 | | 0·79 | |
| 48 | 28 | 24 | 108 | 167 | 12 | 16 | 16 | 60 |
| | 16 | 38 | 36 | 137 | 24 | 6 | 108 | 118 |
| | 4 | 27 | 144 | 180 | | | 64 | 103 |
| Totals | 48 | 89 | 288 | 484 | 36 | 22 | 188 | 281 |
| Ratio $I/N$ | 0·54 | | 0·59 | | 1·64 | | 0·67 | |

(c) *Counts on* Medicago sativa *L.*

A few counts on effectively nodulated plants of lucerne, taken from agar slope cultures are presented in Table 3. Some lucerne plants were found, unlike red clover, to have a marked excess of infections compared with nodules.

These results differ markedly from the report made by McCoy in 1932, where a great excess of infected hairs was observed, in one case with a ratio of 68 to 1 of infected hairs to nodules. However, the difference in the two counts may be related to the method of growing the plants, since McCoy records only 0·25 per cent. infection in lucerne grown in tube culture, compared with 2·5 per cent. in sterilized sand. It seems not unlikely that abrasions to the roots may facilitate entry in the latter case.

## IV. Discussion

These results show that the primary infection of the root of a number of species of clover and of lucerne is no more, or only a little more, than sufficient to produce the number of nodules found thereon.

Some evidence has been produced that nodulation on red clover occurs at discrete foci on the roots (Purchase and Nutman 1957). Such foci may be the disomatic cells described in the elegant studies of Wipf and Cooper (1940).

We may postulate that, with red clover, invasion can only occur in a root hair close to a disomatic "centre" of activity. In the other species examined, entry is clearly more promiscuous, and may indeed correspond to the behaviour of pea and vetch noted by Wipf and Cooper (1940), who observed that "an infection thread may pass through nearly all the cortical cells and cause no stimulation whatsoever. In such cases there are no evidences of either a disomatic cell with its larger nucleus or a group of such cells."

TABLE 3

COUNTS OF NODULES AND INFECTED ROOT HAIRS ON LUCERNE

| Age (days) | No. of Nodules ($N$) | Estimated No. of Infected Hairs ($I$) | Ratio $I/N$ |
|---|---|---|---|
| 28 | 9 | 28 | 3·1 |
|  | 6 | 20 | 3·3 |
|  | 2 | 0 | 0 |
| 48 | 11 | 0 | 0 |
|  | 12 | 4 | 0·3 |
|  | 7 | 8 | 1·1 |
|  | 7 | 4 | 0·6 |
|  | 14 | 132 | 9·4 |
| Totals | 68 | 196 | Mean 2·9 |

More detailed cytological studies might help to present a clearer picture.

Mixtures of strains have been isolated from a single nodule in rather rare instances (Vincent 1954). The double infections, or, more especially, the occurrence of two or more infected hairs per nodule may account for such cases. It still seems possible that entry may be effected by a single bacterium.

## V. Acknowledgments

Microbiology, University of Sydney, with the assistance of Miss Joanne Smith. Thanks are also due to Professor J. M. Vincent and Dr. P. S. Nutman for their criticism of the manuscript.

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# ENVIRONMENT AND SPORULATION IN PHYTOPATHOGENIC FUNGI

## I. MOISTURE IN RELATION TO THE PRODUCTION AND DISCHARGE OF CONIDIA OF PERONOSPORA TABACINA ADAM

### By I. A. M. CRUICKSHANK*

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### Summary

In an investigation into the effects of water relations on sporulation of *Peronospora tabacina* Adam in tobacco leaf disks the following results were obtained:

(i) Both diffusion pressure deficit (D.P.D.) and relative humidity (R.H.) were shown to be of critical importance to sporulation. The threshold values for maximum sporulation were 2·6 atm and 97 per cent. respectively. Minor deviations from these values towards either a higher D.P.D. or a lower R.H. very significantly reduced sporulation intensity.

(ii) Some aspects of the effect of R.H. level × time were analysed.

(iii) It was shown that in addition to the requirement of a minimum period of optimum conditions at a specific time of day for sporulation, there exists also a stage of conidiophore development which is completely dependent on favourable humidity conditions.

(iv) Subminimum periods of optimum humidity on consecutive days were shown to be non-cumulative, and to have no positive effect on sporulation.

(v) Both change in R.H. and mechanical shock were shown to be capable of causing conidial discharge.

These results are briefly discussed in relation to epidemiology of disease development.

## I. INTRODUCTION

Yarwood (1956) has recently reviewed published work concerning the relationship between humidity and sporulation *in vivo* of plant pathogenic fungi. Little quantitative data on this subject has been published and qualitative results in most publications have been limited to field observations under uncontrolled conditions.

Studies of the sporulation of *Peronospora tabacina* Adam have been reported by Clayton and Gaines (1933), Armstrong and Sumner (1935), and Dixon, McLean, and Wolf (1936). These authors have shown that under field, seed-bed, and laboratory conditions sporulation was limited to the range of humidity from atmospheric saturation to slightly below dew-point and that it occurred most abundantly when these conditions occurred at night or over prolonged periods when the sky was overcast.

The mechanism of conidial discharge in *P. tabacina* has been previously studied by Pinkard (1942) who claimed that it was entirely a response to change in humidity.

In a preliminary note (Cruickshank and Müller 1957), a technique was described for the quantitative study of sporulation as a function of individual environmental factors under controlled conditions. This paper reports in greater detail the interaction between water relations and sporulation *in vivo*. An attempt is also made to evaluate the relative importance of humidity changes and mechanical shock in the discharge of conidia of this fungus.

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## II. Materials and Methods

Physiologically similar leaves from uniform glass-house-grown tobacco (*Nicotiana tabacum* L. var. Virginia Gold) plants were used as the host plant material. Prior to the experiments described below the plants were inoculated with a spore suspension of *P. tabacina*. After incubation at high humidity (R.H. > 97 per cent., temp. 20°C) for 48 hr the plants were transferred to a relatively low humidity in a glass-house until symptoms of vegetative development of the pathogen were visible as mild chlorosis of the leaves. At this pre-sporulation stage of symptom development, uniformly infected leaves were detached and disks (15 mm in diameter) were punched from them. These leaf disks were used as the basic leaf units in the quantitative experiments reported.

All experiments were carried out at constant temperature (20°C) and under a standard light intensity (600 lux, fluorescent light) and a 12-hr photoperiod (0600–1800 hr).

In the sporulation experiments, leaf disks were transferred, abaxial surface upwards, to micro-environment chambers (see Cruickshank and Müller 1957). The R.H. of the air above the leaf disks was controlled by glycerol–water mixtures (Carson 1931) in the base of the chambers. Refractive indices of the solutions were measured and the R.H. determined from a standard calibration curve. Where the effect of specific humidity levels was studied the chambers were sealed with glycerol. In all other experiments a high level of humidity (R.H. 98 per cent.) was readily maintained and sealing was unnecessary.

The diffusion pressure deficit (D.P.D.) of the leaf tissue was adjusted by floating leaf disks on mannitol and sucrose solutions contained in "Perspex" cells (15 mm in internal diameter, 5 mm deep) standing in the glycerol–water solutions.

At the termination of each experiment the micro-environment chambers were opened and the leaf disks removed from the "Perspex" cells, drained on filter paper to remove surplus liquid from the adaxial surface, and dropped into 0·5 ml of 50 per cent. ethanol in McCartney bottles. The latter were agitated for $\frac{1}{2}$ min on a "Microid" shaker and the concentration of the spore suspension measured by counting standard fields in a haemocytometer chamber. Morphological examination showed that only mature spores were released by this method. Serial transfer and agitation tests of disks also showed that more than 90 per cent. of spores were released into solution during the first agitation. The spore concentration was taken as a measure of the sporulation intensity.

In the experiments on conidial discharge the following modifications to the basic technique were made. Water–agar blocks (15 mm in diameter, 2 mm thick) replaced the mannitol solutions. The leaf disks were placed with their adaxial surface against the agar blocks which were inverted and attached by surface tension to the inside of petri-dish lids. One disk was placed centrally within each lid. A standard microscope slide smeared with glycerol was supported between the leaf disk and the glycerol solution as a spore trap. The intensity of spores caught on the slides was assessed by microscopic examination on a 0–10 scale (0 = no spores, 10 = heavy spore deposit).

Leaf disks were randomized and six to nine replications were used in each experiment. All experiments were repeated in time and results from representative experiments are given. The means of the spore concentrations were calculated as percentages of the maximum spore concentration within experiments and were plotted against treatment in the figures presented.

## III. Experimental

### (a) Sporulation

#### (i) Effect of Diffusion Pressure Deficit of Host Tissue

To study the effect of D.P.D. of leaf tissue on sporulation intensity the D.P.D. of the leaf disks was adjusted from 2·6 to 20·7 atm by floating them on solutions of sucrose and mannitol ranging from 0·1 to 0·8m. Zero D.P.D. was obtained using glass-distilled water only. The R.H. was kept constant (98 per cent.) and experiments were run over a period of 24 hr.

The possibility of toxicity of either sucrose or mannitol solutions influencing the viability of the pathogen appeared unlikely as spores of *P. tabacina* were successfully germinated on 0·1–0·6m solutions of both compounds. The difference between response of disks floated on mannitol and sucrose was probably attributable to slight permeability of the host cells to sucrose.

The results of this experiment are presented in Figure 1. The shape and slope of the response curve shows that maximal sporulation occurs at pressure deficits below 2·6 atm and that with increasing D.P.D. sporulation intensity rapidly decreases until complete inhibition is reached at 20·6 atm. The first and most significant drop in sporulation intensity appeared to be independent of permanent wilting as it occurred at a D.P.D. value well below that of the osmotic concentration* of tobacco leaf tissues.

#### (ii) Effect of Relative Humidity of the Atmosphere

The relationship between sporulation and R.H. of the air immediately adjacent to the adaxial leaf surface was studied using micro-environment chambers. The proportion of glycerol in glycerol–water mixtures was varied to give vapour pressures equivalent to 100, 99, 98, 97, 96, 94, 92, and 90 per cent. R.H. The D.P.D. was kept constant (2·6 atm) during the 24-hr duration of the experiment. Results are presented in Figure 2. An analysis of the data using the transformation $\log (x+10)$ showed that significant ($P<0.001$) reduction in sporulation intensity occurred between the 97 and 96 per cent. R.H. levels. There was no significant difference in sporulation intensity between 97 and 100 per cent. R.H. The latter could possibly have been due to incomplete control of R.H. over this humidity range owing to slight temperature fluctuation ($\pm0.5°C$). As the R.H. levels dropped towards 90 per cent. the sporulation intensity rapidly approached zero.

#### (iii) Sporulation in Relation to Time

In these experiments an attempt was made to study the development of conidia with time under optimal conditions and to determine whether a relationship existed

*Osmotic concentration of tobacco leaf 8–8·5 atm (cryoscopic method).

between time of onset of optimal humidity conditions, time of initiation of conidia formation, and final intensity of sporulation.

Water relationships were kept constant during these experiments (R.H. 98 per cent., D.P.D. 2·6 atm).

(1) *Development of conidia under optimal conditions.*—Conidial formation with time was studied by setting up 180 leaf disks under optimal conditions and removing at hourly intervals samples of nine disks for measurement of sporulation intensity. The experiment was initiated at 1300 hr and completed at 0900 hr. Sporulation was first detected at 0300 hr. The results presented in Figure 3 show that there is a very short interval of time between the first appearance of conidia and the attainment of the maximum level of sporulation intensity.
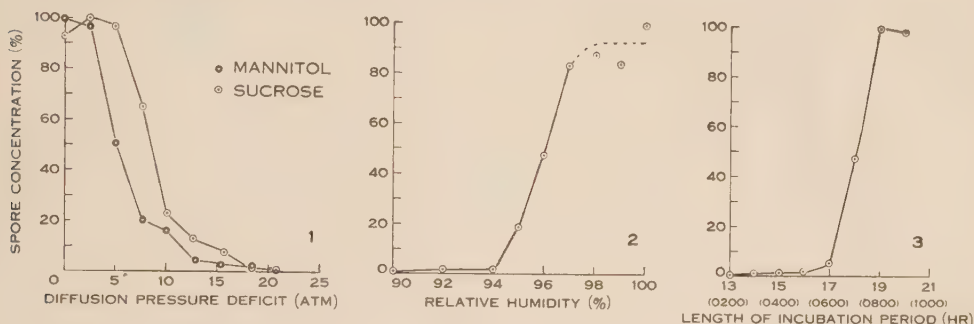


Fig. 1.—Relationship between diffusion pressure deficit of host tissue and sporulation.
Fig. 2.—Relationship between relative humidity of air and sporulation.
Fig. 3.—Sporulation response under optimal conditions. Time of day is given in parenthesis.

(2) *Relationship between humidity, time, and intensity of sporulation.*—Blocks of 30 leaf disks were set up in micro-environment chambers at 1400, 1800, 2200, 2300, 2400, 0100, 0200, and 0300 hr. Samples of six leaf disks were taken for measurement from each block at 0400, 0500, 0600, 0700, and 0800 hr. Later samples were omitted since preliminary tests had shown that normally no significant increase in sporulation intensity occurred after this time (see Fig. 3).

The results presented in Figure 4 illustrated three important points. Firstly, no simple relationship existed between onset of optimum humidity conditions and time of sporulation. Secondly, a minimum period of 3 hr of optimum humidity prior to 0500 hr was necessary for maximum sporulation to occur assuming that conditions remained optimal until 0800 hr. Finally, an analysis of the data using a suitable transformation ($Y = \log (x+10)$) indicated no significant difference in sporulation intensity at 0800 hr irrespective of the length of exposure of the leaf disks to optimum R.H. On the basis of the $t$-test, however, the sporulation resulting from the treatment initiated at 0300 hr appeared to be significantly lower ($P = 0.05$) than all but one of the previous treatments. This latter test indicates that although conditions were satisfactory for sporulation to be initiated, there was barely adequate time for the maximum sporulation intensity to be reached during the duration of this experiment.

(iv) *Effect of Change in Humidity from Optimum to Suboptimum Level during Sporulation*

Since the above experiments had shown that humidity conditions must be satisfactory over a minimum period prior to sporulation for sporulation to occur, this

experiment was designed to test whether, given the above conditions, sporulation could be inhibited once it had begun by lowering the R.H. during and subsequent to the first appearance of conidia.

Leaf disks were placed under optimal conditions (R.H. 98 per cent., D.P.D. 2·6 atm) in micro-environment chambers at 1300 hr. From 2300 to 0600 hr inclusive nine leaf disk samples were transferred at hourly intervals to chambers at suboptimal humidity (R.H. 90 per cent., D.P.D. 2·6 atm). All disks were harvested at 0800 hr
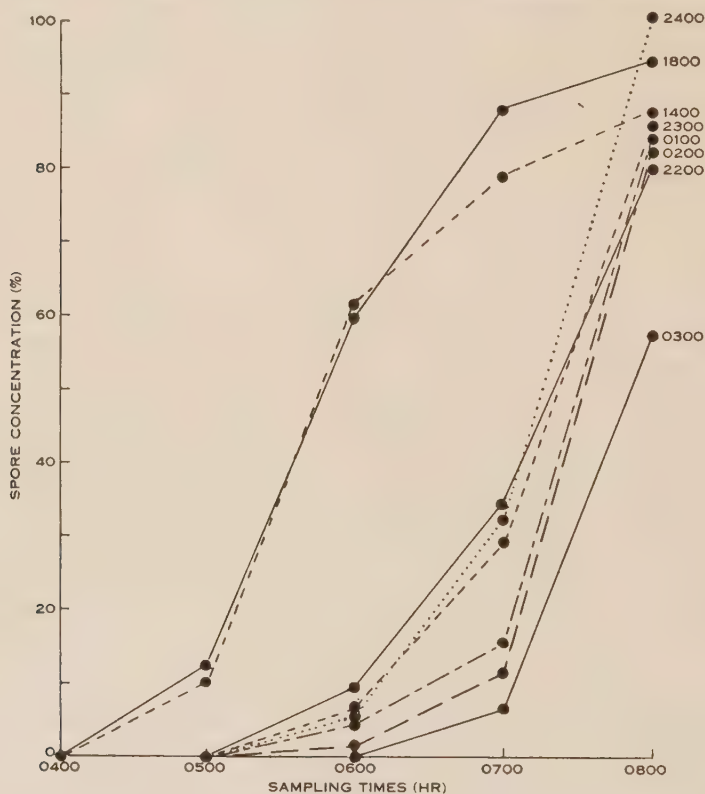


Fig. 4.—Relationship between onset of optimum relative humidity, first appearance of mature conidia, and final sporulation intensity. Treatment initiating times are indicated for each graph.

and sporulation intensity measured. The results presented in Figure 5 show that a change from optimal to suboptimal R.H. during the early stages of sporulation inhibits its further development but that, when the growth of the reproductive structures had reached a certain stage in their development, maturation occurs irrespective of subsequent changes in the R.H. Intermediate conditions also occur where sporulation, although not completely inhibited, is significantly reduced in intensity. The latter is probably due to unequal development within the conidiophore population.

(v) *Effect of Subminimal Periods of Optimal Humidity on Consecutive Days*

Preceding experiments showed that the most important time of day in relation to humidity and sporulation was between 2400 and 0600 hr. It was known from these

experiments that single subminimal periods of optimal humidity prior to 2400 hr were not inducive to sporulation but no data existed on the cumulative effect of subminimal periods of high humidity.

Optimum and suboptimum levels of humidity were similar to those given in Section III(a)(iv).  Leaf disks were maintained under optimal conditions for periods of 3, 6, 9, 12, and 24 hr from 1200 hr on each of 4, 4, 3, 2, and 1 days respectively.  Each day the disks were transferred for the balance of the 24 hr to the suboptimal humidity level.  Other conditions remained unchanged.  From Figure 6 it is clear that intermittent high humidity during the afternoon and evening hours is non-cumulative in its effect.  Potential sporulation was not, however, affected by the treatments as continuation of the incubation period for a further 24 hr at 98 per cent. R.H. resulted in each case in normal sporulation.
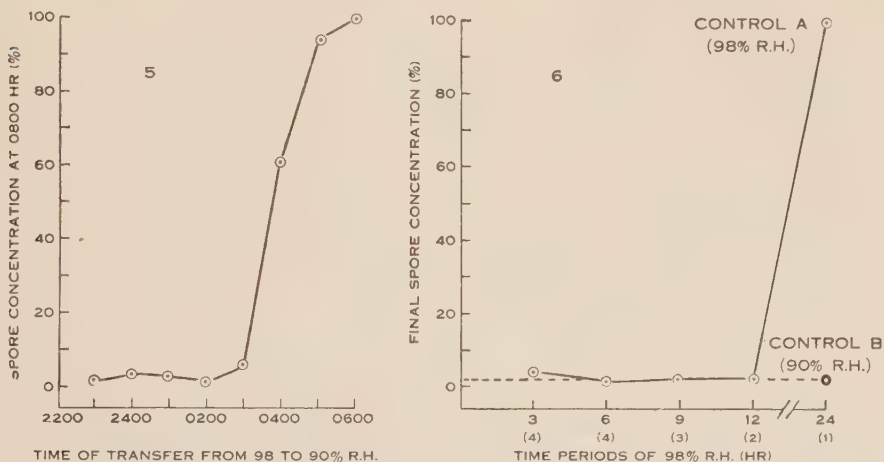


Fig. 5.—Effect on final sporulation intensity of change in relative humidity during sporulation.

Fig. 6.—Effect of subminimal periods of high humidity (R.H. = 98 per cent.) on sporulation.  Number of days of each period is given in parenthesis.

## (b) Conidial Discharge

Using the agar-block technique described above, leaf disks were set up under optimum conditions for sporulation for a period of 24 hr.  The following treatments were then carried out and the intensity of spore discharge estimated.

(i) *Change of Relative Humidity*.—A second set of petri-dish bases were partly filled with glycerol–water mixtures designed to produce vapour pressures equivalent to 98, 90, 80, 70, 60, and 50 per cent. R.H.  Glycerol slides were placed in position as described above and petri-dish lids carrying the sporulating leaf disks were rapidly transferred from the optimum humidity conditions to the lower R.H. series.  The lids were left in the new positions for 1 hr.  Six leaf disk replications were used.

(ii) *Mechanical Shock*.—The R.H. was maintained constant at 98 per cent.  Glycerol slides were set up in the petri dishes prior to the initial 24-hr incubation period.  The dishes were then transferred, with adequate protection against disturb-

ance in transit, to an apparatus in which a weight (1 g) was mounted centrally over the petri dish directly above the leaf disk. The weight was dropped on to the lid from heights of 5, 10, 20, 40, and 60 mm. Six leaf disk replications were used. Results of both experiments in Section III(b) are presented in Figures 7(a) and 7(b). From a comparison of the slopes of the curves it appeared that both changes in R.H. of the air adjacent to the sporulating surface and the magnitude of the mechanical shock suffered by the infected leaf were important contributing factors in the discharge of spores. This response increased with increasing magnitude of humidity change and size of mechanical shock.
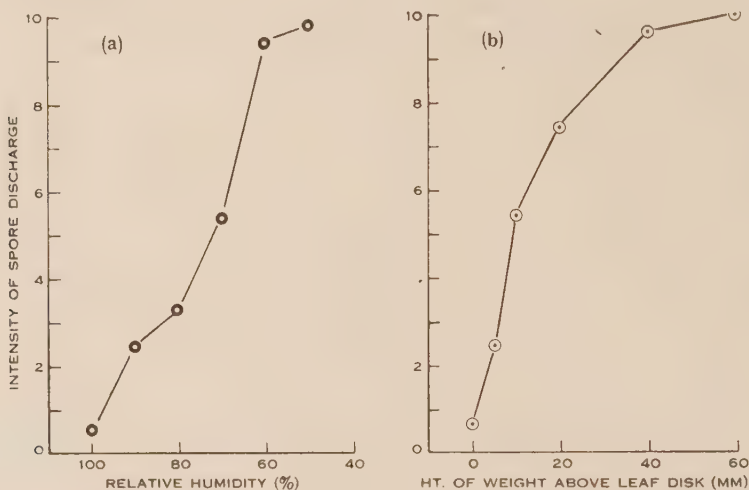
Fig. 7.—Intensity of spore discharge in relation to: (a) change in relative humidity; (b) mechanical shock (R.H. constant, 98 per cent.; magnitude of weight, 1 g).

## IV. Discussion and Conclusions

Water relations, as shown by earlier workers, proved to be an important factor in the environment in relation to the phenomenon of sporulation in *P. tabacina*. While the general relationship between atmospheric humidity and sporulation has long been observed, the critical relationship between sporulation intensity and D.P.D. of the host tissue and R.H. of the air in the immediate vicinity of the abaxial leaf surface have not been previously reported. The leaf-disk technique has enabled the effect of hydrostatic pressures inside and vapour pressures outside the leaf tissues to be studied independently of each other while maintaining other conditions constant.

Threshold values of the D.P.D. and R.H. for maximum sporulation were 3–4 atm, and 97 per cent. respectively. Small increases in D.P.D. (Fig. 1) or decreases in R.H. (Fig. 2) very significantly decreased sporulation intensity. These analyses have demonstrated the critical sensitivity of the sporulation mechanism in *P. tabacina* to both internal and external water relations.

Wolf and McLean (1940) stated that under natural conditions conidia were produced at daybreak each morning. An analysis of the progressive development of

fructification in terms of conidia production with time under optimum conditions (Fig. 3) confirmed this claim. Similar experiments to the one reported showed that the position of the curve may move a little to the left or right but that the shape and slope are constant. On the basis of morphological observations at the time of sampling (Cruickshank, unpublished data) it was apparent that only fully developed spores were released into the alcohol solution by agitation. Conidiophores were macroscopically visible with immature conidia attached in samples taken up to 3 hr prior to the general detection (50 per cent. level of intensity) of fully developed spores. These observations along with the spore measurement data presented suggest that the incubation period required for sporulation may be divided into a relatively long one of vegetative growth of conidiophores and a short period of conidia formation and maturation.

Yarwood (1937) has suggested that light may exert a controlling influence on sporulation in certain downy mildews. The coincidence of conidia formation and maturation with the onset of light suggested it may play a role in determining the time of sporulation. Other results obtained confirmed that light was involved but that its effect was more indirect than previously reported.

The minimum period of optimum conditions required for sporulation of infected leaf has not previously been reported. Studies of sporulation in relation to varying periods of optimum conditions showed that 3 hr prior to 0500 hr were necessary (Fig. 4). However, when the results of Figures 4 and 5 are considered together it becomes apparent that, as the 0300 hr treatment would have matured irrespective of humidity change, if the experiment had been continued beyond 0800 hr, the minimum period of optimum conditions does not need to occur prior to 0500 hr. Infected leaf disks cut from plants grown under natural day length conditions could not be induced to sporulate at any other time of the day. A 12-hr difference (1400–0200 hr) in length of optimum conditions caused only a 2-hr difference in time of onset of conidia formation (10 per cent. level of intensity) and no significant difference in the final intensity of sporulation.

Change in R.H. during the hours of 2300 hr and 0600 hr from 98 to 90 per cent. (Fig. 5) showed that in addition to the requirement of a minimum period of optimum conditions at a specific time of day for sporulation, there exists also a stage in conidiophore development that is completely dependent on optimum humidity conditions. A drop in R.H. to 90 per cent. before this stage resulted in inhibition of conidia formation. If conidiophore development had progressed beyond this stage when the humidity was altered conidia formation and maturation continued to occur irrespective of the change in R.H.

The effect of high levels of humidity for periods of varying length from 1200 to 2400 hr on consecutive days was shown (Fig. 6) to be non-cumulative and to have no positive significance to the phenomenon of sporulation. The potential sporulation capacity of the infected tissues was not, however, affected as normal sporulation occurred when the necessary conditions were provided.

Pinkard (1942) has described the mechanism of conidial discharge in *P. tabacina* and attributed this phenomenon to R.H. changes. Yarwood (1943) in the course of studies on *P. destructor* concluded that conidia were released by changes in humidity and mechanical effects. In the present analysis it is shown that either change in

humidity or mechanical shock (Figs. 7(a) and 7(b)) may be involved in conidial discharge. Although under field conditions it would be difficult to separate these two factors, it was shown in the experiments described that each may act independently of the other and that their effect increases with the magnitude of the change in R.H. or the size of the shock. Spore dispersal was progressive and not simultaneous as reported by Wolf and McLean (1940).

Müller and Haigh (1953) and Waggoner (1956) have recently shown mathematically the relationship between sporulation intensity and the epidemiology of plant disease. The studies reported in this paper emphasize the great importance of favourable water relations to the sporulation *in vivo* of *P. tabacina* and show that minor deviations from the optimum humidity–time balance described will either extend the length of the incubation period of the development cycle or significantly decrease the intensity of sporulation. Either or both of these results would greatly affect the epidemiology of blue mould (*P. tabacina*) as it would result in a decrease in the speed of build-up of the spore population. The results presented may also explain much of the variation in blue mould that occurs both within and between tobacco crops (Angell and Wark 1955).

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# THE WATER RELATIONS OF *VIBRIO METCHNIKOVI* AT 30°C

By Betty J. Marshall* and W. J. Scott*

*Summary*

For a strain of *Vibrio metchnikovi* the rate of growth in liquid media and the number of colonies forming on solid media were both greatly increased by reducing the water activity ($a_w$) from c. 0·999 to 0·995.

Below c. 0·995 $a_w$ both the rates of growth and colony numbers were greater in the presence of salts than in the presence of sucrose or glucose.

## I. Introduction

*Vibrio metchnikovi* is a pathogen for birds and some laboratory animals. In several respects it is similar to the cholera vibrio and, like this organism, it is relatively susceptible to drying (Annear 1956). The organism was found to grow luxuriantly in brain heart infusion, but in nutrient broth containing 5 g peptone and 3 g of meat extract per litre it grew very slowly and at times only after a prolonged lag phase. As the former medium, containing 37 g dry matter per litre, had a water activity ($a_w$) of 0·993, and the latter an $a_w$ of c. 0·999 (Scott 1953), it was decided to examine the effect of adding various solutes to nutrient broth. The addition of small quantities of salts to this medium permitted greatly increased rates of growth and therefore the effects of various levels of $a_w$ were studied in more detail. This paper reports the effects of $a_w$ on the rate of growth and on the plate count of this organism. A wider discussion of the effect of $a_w$ on microbial growth has been given elsewhere (Scott 1957).

## II. Methods

The methods for controlling $a_w$ by the addition of solutes, and for determining rates of growth in rocking T-tubes, were as described previously (Scott 1953). Two basal media each of c. 0·999 $a_w$ were used. These were nutrient broth and brain heart infusion containing respectively 8 and 5 g of dry matter per kilogram of water. The solids for the brain heart infusion were obtained by freeze-drying a large batch prepared according to the formula in the Difco manual (9th Ed.). Solid media of virtually the same $a_w$ were made by dissolving 8 g of agar per kilogram of water in the corresponding liquid media.

For the plate counts, dilutions were made in sodium chloride solution (0·995 $a_w$) and, for some experiments, in solutions of potassium chloride (0·995 $a_w$) or in mixed electrolytes (NaCl:KCl:Na$_2$SO$_4$ : : 5: 3: 2 moles, 0·995 $a_w$). Plates were inoculated with 1 ml of the diluted suspension and, for each medium, triplicate plates were poured with c. 15 ml of the melted agar cooled to 45°C. The water in the inoculum was sufficient to increase the $a_w$ of the agar at 0·970 $a_w$ by almost 0·002 and at 0·980 $a_w$ by c. 0·001. For media of higher $a_w$ the change due to the water in the inoculum was very much less. In experiments in which different diluents were compared (Table 1) plates

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were inoculated with only 0·1 ml of the diluted suspension for each 15 ml of agar. This ensured that the diluent with the greatest solute concentration did not depress the $a_w$ of the agar medium by more than 0·0002.

Plates were counted after incubation for 2 and 5 days at 30°C. To reduce evaporation from the medium, and, at the same time, to provide oxygen, the plates were incubated in metal containers with loosely fitting lids.

The culture studied was forwarded by Dr. D. I. Annear.

## III. Results

### (a) Growth in Liquid Media

The rates of growth in nutrient broth and brain heart infusion each adjusted to several levels of $a_w$ with a number of solutes are shown in Figures 1 and 2 respectively. The values plotted are the means of two to four determinations. Figure 1 shows that
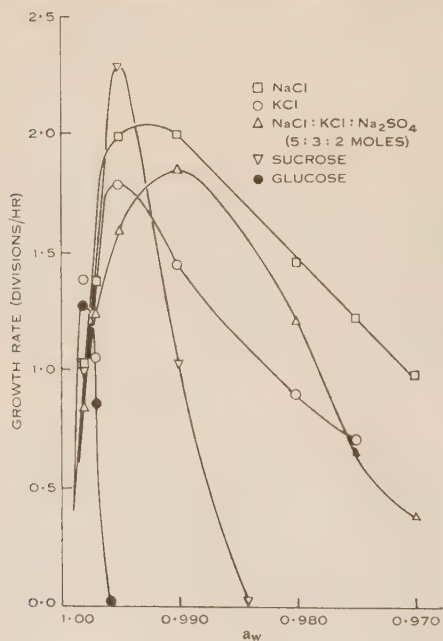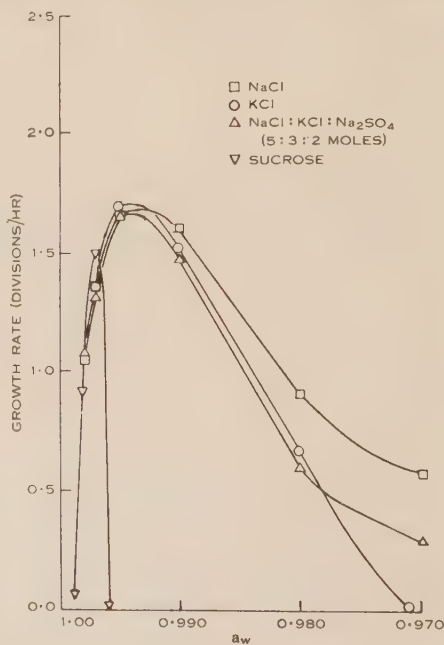


Fig. 1                    Fig. 2

Figs. 1 and 2.—Relation between rate of growth at 30°C and $a_w$ for *Vibrio metchnikovi* in nutrient broth (Fig. 1) and in brain heart broth (Fig. 2) with added solutes.

all solutes tested caused a sharp increase in the rate of growth as the $a_w$ was reduced below 0·999. For sodium and potassium chlorides and sucrose the maximum rates of growth occurred at 0·995 $a_w$. For the salts mixture the greatest rate of growth occurred at 0·990 $a_w$, whereas with glucose it was at 0·998 $a_w$. The inhibition by glucose at less than 0·997 $a_w$ has not been explained. With the brain heart infusion (Fig. 2) similar trends with the various solutes were revealed, although stimulation in sucrose media was now greatest at 0·997 $a_w$, and in media containing added glucose growth was not

observed at all. Although the maximum rates of growth shown in Figure 2 are some-what less than those in Figure 1, a greater rate of 2·4 divisions/hr was observed in brain heart infusion of 0·993 $a_w$ and containing 37 g of dry matter per kilogram of water. This is the concentration of nutrients usually recommended for this medium, and is over 7 times the concentration used in the experiments summarized in Figure 2. For both the basal media the greatest rates of growth between 0·990 and 0·970 $a_w$ were obtained when sodium chloride was the added solute.

### (b) The Plate Count

The numbers of colonies which developed on nutrient agar adjusted to various levels of $a_w$ with different solutes are shown in Figures 3 and 4. The inocula consisted
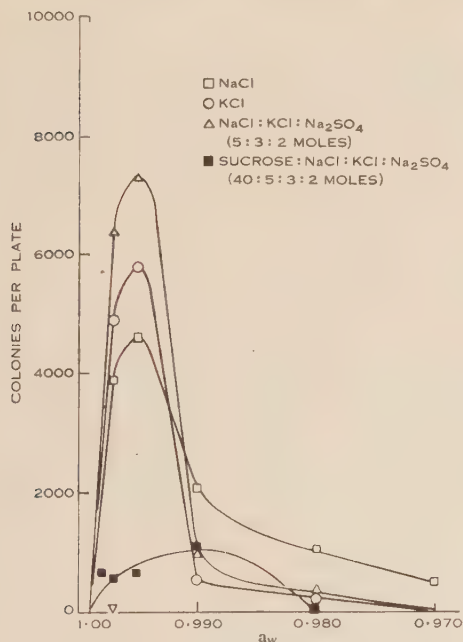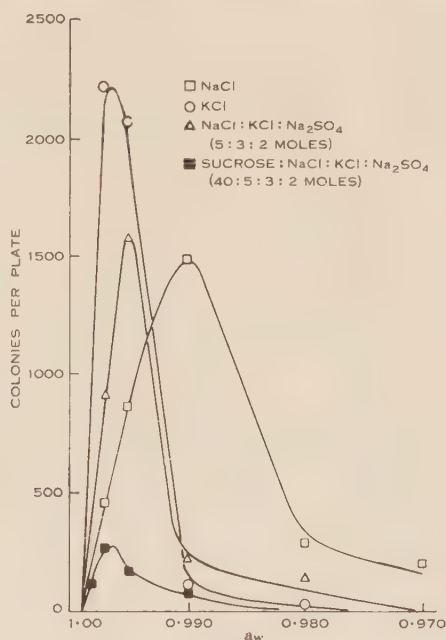


Fig. 3                                        Fig. 4

Fig. 3.—Relation between plate count and $a_w$ of nutrient agar for *Vibrio metchnikovi* grown at 0·995 $a_w$. Counts are means for triplicate plates inoculated with $10^{-6}$ ml of a culture grown for 6 hr at 30°C in nutrient broth adjusted to 0·995 $a_w$ with KCl (0·121m).

Fig. 4.—Relation between plate count and $a_w$ of nutrient agar for *Vibrio metchnikovi* grown at 0·975 $a_w$. Counts are means for triplicate plates inoculated with $10^{-6}$ ml of a culture grown for 18 hr at 30°C in nutrient broth adjusted to 0·975 $a_w$ with NaCl (0·727m).

of replicate 1-ml aliquots of the same diluted suspension. It is clear that the addition of some solutes has had a large influence on the number of colonies which appeared. For instance, for the experiment summarized in Figure 3 only 24 colonies developed on the control basal medium of 0·999 $a_w$ whereas over 7000 colonies appeared when the $a_w$ was reduced to 0·995 with the salts mixture. In contrast to its effects on the rate of growth, sucrose has had little effect on the number of colonies. No colonies appeared

when $a_w$ was adjusted with glucose, but over 1000 colonies were formed when $a_w$ was controlled by a sucrose–salts mixture. For this latter mixture the maximum count occurred at $0.990\ a_w$ whereas with the salts mixture the colony count was much greater at $0.995\ a_w$. For an inoculum grown at $0.975\ a_w$ results in Figure 4 show similar large effects of electrolytes. No colonies occurred on the basal medium but over 2000 developed when the agar was adjusted to $0.995$ or $0.997\ a_w$ with potassium chloride. Addition of a salts mixture or sodium chloride produced media giving counts of c. 1600 and 1500 at $0.995$ and $0.990\ a_w$ respectively. When the $a_w$ was adjusted with sucrose or glucose no colonies were found, but 267 developed on agar adjusted with the sucrose–salts mixture to $0.997\ a_w$. It may be seen that although grown at $0.975\ a_w$ the

TABLE 1

EFFECT ON THE PLATE COUNT OF VIBRIO METCHNIKOVI OF VARIATIONS IN THE $a_w$ OF THE PLATING MEDIUM AND OF THE DILUENT

The values represent the mean number of colonies on triplicate plates each inoculated with $10^{-6}$ ml of a saline broth culture. The culture consisted of nutrient broth adjusted to $0.975\ a_w$ with NaCl, and incubated for 18 hr at 30°C

| $a_w$* of Nutrient Agar Used for Plating Medium | $a_w$* of Diluent | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0·999 | 0·997 | 0·995 | 0·990 | 0·980 | 0·975 | 0·970 |
| 0·999 | 1 | 1 | 3 | 5 | 1 | 2 | 3 |
| 0·997 | 45 | 1150 | 340 | 260 | 530 | 680 | 700 |
| 0·995 | 140 | 1920 | 1570 | 1860 | 1690 | 1610 | 2020 |
| 0·990 | 29 | 580 | 1630 | 990 | 650 | 1420 | 1530 |
| 0·980 | 0 | 990 | 810 | 1040 | 450 | 540 | 1430 |
| 0·970 | 0 | 39 | 220 | 79 | 300 | 330 | 400 |

*Controlling solute NaCl.

maximum count occurred on media adjusted to $a_w$ between $0.990$ and $0.997$. Sodium chloride was the only solute permitting colony development at $0.970\ a_w$, but in neither experiment did this solute lead to the greatest number of colonies at $0.995\ a_w$. Results similar to those shown in Figure 4 have been obtained also when dilutions were made in solutions of potassium chloride or of a mixture of sodium and potassium chlorides and sodium sulphate at $0.995\ a_w$.

Although the results in Figure 4 indicate that cells grown at $0.975\ a_w$ gave the greatest plate count at c. $0.995\ a_w$, the result may have been a consequence of diluting the cells at $0.995\ a_w$ prior to plating. Accordingly, cells were diluted in sodium chloride solutions at seven concentrations between $0.999$ and $0.970\ a_w$ and subsequently plated on six agar media covering the same $a_w$ range. Results of a typical experiment are given in Table 1, from which it may be seen that for all seven diluents the greatest numbers of colonies developed when the $a_w$ of the agar was close to $0.995$. In other words, the probability that a cell would form a colony was greatest at c. $0.995\ a_w$ even for cells grown and diluted in more concentrated solutions of sodium chloride. It is

shown also in Table 1 that dilution in 0·999 $a_w$ sodium chloride results in over 90 per cent. mortality. Similar trends have been shown in other experiments, using cells harvested during the logarithmic growth phase as well as during the stationary phase. There is, however, reason to believe that different cultures grown under apparently similar conditions may nevertheless vary in their susceptibility to lysis on dilution. In one exceptional experiment with stationary phase cells grown at 0·975 $a_w$ it was found that diluents of 0·997 and 0·995 $a_w$ were, like the 0·999 $a_w$ diluent, markedly destructive, whereas dilution in more concentrated solutions caused no destruction. In further tests with the same series of diluents and agar media, destruction was confined to the 0·999 $a_w$ diluent.

## IV. Discussion

Comparison with results obtained for some other bacteria shows that *Vibrio metchnikovi* is relatively exacting in its osmotic and ionic requirements. For instance, in nutrient broth reduction of the $a_w$ from 0·999 to 0·995 caused a five-fold increase in the rate of growth (Fig. 1), whereas with salmonellae (Christian and Scott 1953) and *Staphylococcus aureus* (Scott 1953) the same change in $a_w$ increased the growth rate by only 10 and 20 per cent. respectively. The contrast between these bacteria and *V. metchnikovi* is even more striking when the relation between $a_w$ and the plate count is considered. For salmonellae and staphylococci the plate count showed a considerable independence of $a_w$ whereas for *V. metchnikovi* the probability that a cell would form a colony was extremely sensitive to relatively minor variations in the osmotic and ionic conditions of the plating medium.

Although the present experiments do not explain the osmotic sensitivity of *V. metchnikovi*, it is of interest to point out that some other organisms appear to have comparable properties. For instance, Koser, Breslove, and Dorfman (1941) reported that the growth of several species of *Brucella* was greatly stimulated by the addition of several electrolytes including sodium chloride. The calculated $a_w$ of their basal medium No. 4 was close to 0·999, and the optimum concentration of sodium chloride reduced this to between 0·995 and 0·993.

The rate of growth of all the *Brucella* strains was increased by the addition of salts to the basal medium, and two strains of *Brucella suis* did not grow unless the amount of added sodium chloride was sufficient to reduce the $a_w$ to about 0·997. Mager (1955) has reported that the growth of *Neisseria perflava* and *Pasteurella tularensis* was stimulated by various salts and sugars, the optimum concentration in each case corresponding to 0·997–0·995 $a_w$. Rodwell (1956) has shown that the growth of *Asterococcus mycoides* was sensitive to the tonicity of the medium when serum was replaced by other factors. These few organisms which are osmotically sensitive are also pathogenic to animals, and it may be that the parasitic existence in the closely controlled environment of the host has led to a loss of properties conferring resistance to osmotic change. With *A. mycoides* the lack of a rigid cell wall is an obvious factor which may lead to osmotic fragility. Further work would be needed to show whether the properties of the cell wall are a cause of the requirements manifested by *V. metchnikovi*, and the abovementioned *Brucella*, *Neisseria*, and *Pasteurella* strains.

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# THE INTERACTION OF pH, TONICITY, AND ELECTROLYTE CONCENTRATION ON THE MOTILITY OF FOWL SPERMATOZOA

By R. G. Wales* and I. G. White*

*Summary*

The motility of fowl spermatozoa has been studied *in vitro* under various modifications of pH, osmotic pressure, and chemical composition of diluents. The glucose and sodium chloride content of the diluents has been varied to give tonicities ranging between that of 0·45 and 1·8 per cent. sodium chloride. These diluents were buffered with citric acid–disodium phosphate, sodium phosphates, or sodium carbonate–bicarbonate mixtures which were equally innocuous.

Fowl spermatozoa showed maximum motility at pH 7 and the pH of fowl semen was $7·6 \pm 0·42$. At a pH of 8·7–8·9 partial replacement of sodium chloride by glucose was favourable in hypotonic, isotonic, and hypertonic diluents.

Under all conditions hypotonic diluents were more deleterious than hypertonic ones of similar composition and the spermatozoa were able to partially adapt themselves to extreme osmotic pressures.

## I. INTRODUCTION

Hydrogen ion concentration and tonicity are two of the most important factors influencing the survival of mammalian spermatozoa; the effect of electrolyte, in the form of sodium chloride, has, however, most probably been overrated by early workers.

The first systematic studies of all three factors was made by Emmens (1947, 1948) who observed the motility of rabbit spermatozoa at various pH levels with diluents of different tonicity and chemical composition. The optimal pH for motility of rabbit spermatozoa was found to be about 7 but they were at least partially motile between pH 5 and 10. Both hypotonic and hypertonic diluents were deleterious at all pH levels but the relative effects of hypo- and hypertonicity varied with pH. Thus in acid or neutral solutions the spermatozoa were more susceptible to hypo- than to hypertonicity, but in alkaline media the situation was reversed. Contrary to what might have been expected from the early literature (see Anderson 1945) motility was not significantly affected by changes in the proportion of sodium chloride and glucose in buffered diluents of the same tonicity, except above pH 9.

More recently these studies have been extended to ram, bull, and human spermatozoa (Blackshaw and Emmens 1951). Ram and bull spermatozoa showed optimal motility at about pH 7 and human spermatozoa at about pH 8·5; the spermatozoa of all three species were at least partially motile within a pH range of 5–10 and bull spermatozoa were not completely immobilized even at pH 4·4. In these latter species hypertonic solutions were less harmful than hypotonic media at all pH levels and, furthermore, the relatively slight adverse effect of hypertonicity could be diminished by partial replacement of sodium chloride by glucose at alkaline pH's.

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There appears to be an almost complete lack of information on the effect of these factors on fowl spermatozoa, and it is the purpose of this paper to present similar studies using the spermatozoa of this species.

## II. MATERIAL AND METHODS

Fowl semen was obtained by abdominal massage (Burrows and Quinn 1939). Only apparently normal specimens of good initial motility were employed, usually pooled with at least one other ejaculate.

The diluents used were glucose and sodium chloride mixtures buffered with (a) citric acid and disodium phosphate, (b) mono- and disodium phosphates, or (c) sodium bicarbonate–carbonate. The tables of Kolthoff and Rosenblum (1937) and

TABLE 1
COMPOSITION OF DILUENTS

| Diluent | Relative Tonicity (0·9% NaCl = 100) | Glucose (%) | Added NaCl (expressed as %) at Nominal pH: | | | | |
|---|---|---|---|---|---|---|---|
| | | | 4·0 | 5·5 | 7·0 | 8·5 | 10·0 |
| A | 50 | 2·00 | 0·00 | 0·00 | 0·00 | 0·00 | 0·00 |
| B | 100 | 2·00 | 0·46 | 0·46 | 0·42 | 0·46 | 0·43 |
| C | 150 | 2·00 | 0·91 | 0·91 | 0·87 | 0·91 | 0·88 |
| D | 200 | 2·00 | 1·36 | 1·36 | 1·32 | 1·36 | 1·33 |
| E | 50 | 1·00 | 0·17 | 0·17 | 0·13 | 0·17 | 0·14 |
| F | 100 | 3·75 | 0·17 | 0·17 | 0·13 | 0·17 | 0·14 |
| G | 150 | 6·55 | 0·17 | 0·17 | 0·13 | 0·17 | 0·14 |
| H | 200 | 9·30 | 0·17 | 0·17 | 0·13 | 0·17 | 0·14 |

Vogel (1943) were used as a guide in preparing the buffers, the proportions of the constituents—which were all at a final concentration of 0·02M—being carefully adjusted to give the desired pH. Details of the composition of the diluents are given in Table 1, the relative tonicity being calculated on the assumption that all electrolytes were completely dissociated. Due to dissociation into varying numbers of ions the tonicity of the buffer mixtures varied slightly at different pH levels and this was compensated by adjusting the sodium chloride concentration of the diluent. All diluents were stored in a deep-freeze and the pH checked with a glass electrode meter before and after each experiment. The actual pH of suspensions sometimes differed slightly from the intended pH, particularly towards the end of some experiments, and the extent of this variation is shown in the tables by the standard deviation.

Semen was diluted 1 in 20 in small tubes and kept at room temperature. For the determination of motility, a drop of spermatozoal suspension was placed on a glass slide and examined under the microscope within a few minutes. Motility was scored by the system of Emmens (1947) at $\frac{1}{2}$, $1\frac{1}{2}$, $2\frac{1}{2}$, 4, and $5\frac{1}{2}$ hr unless otherwise

stated. Full motility was rated as 4 and complete immotility as zero, but in presenting results the actual scores have been multiplied by 4, since quarter-grades were frequently used.

The motility index referred to in the tables is the sum of the motility scores × 4 for each ejaculate over the experimental period. This has been used as unit observation (see Emmens 1948) for the analyses of variance which are presented in summary form.

TABLE 2

MOTILITY INDICES FOR FIVE FOWL EJACULATES IN DILUENTS CONTAINING VARIOUS BUFFERS

| pH | Buffer | Diluent B | | | | | | Diluent F | | | | | | Grand Totals |
|----|--------|-----------|---|---|---|---|-------|-----------|---|---|---|---|-------|--------------|
| | | Ejaculate No. | | | | | Total | Ejaculate No. | | | | | Total | |
| | | 1 | 2 | 3 | 4 | 5 | | 1 | 2 | 3 | 4 | 5 | | |
| 6·1 | Citric acid–phosphate | 10 | 14 | 29 | 27 | 7 | 87 | 16 | 13 | 14 | 17 | 3 | 63 | 150 |
| | Phosphate | 15 | 16 | 24 | 37 | 6 | 98 | 17 | 18 | 20 | 21 | 7 | 83 | 181 |
| 8·4 | Carbonate–bicarbonate | 16 | 26 | 30 | 52 | 60 | 184 | 57 | 51 | 55 | 55 | 64 | 282 | 466 |
| | Phosphate | 28 | 41 | 39 | 50 | 64 | 222 | 45 | 53 | 24 | 57 | 64 | 243 | 465 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Variance Ratios | |
|---------------------|--------------------|-----------------|--|
| | | Citric Acid–Phosphate v. Phosphate | Carbonate–Bicarbonate v. Phosphate |
| Between diluents | 1 | 6·3 | 9·6* |
| Between buffers | 1 | 4·0 | 0·0 |
| Between ejaculates | 4 | 19·2** | 7·1* |
| Interactions: | | | |
| Diluent × buffer | 1 | 0·3 | 4·0 |
| Buffer × ejaculate | 4 | 0·5 | 0·7 |
| Diluent × ejaculate | 4 | 4·3 | 1·8 |
| Residual | 4 | 12 | 74 |

*$P < 0.05$.        **$P < 0.01$.

## III. RESULTS

### (a) Preliminary Tests

No one buffer would cover the wide pH range (4–10) required and preliminary tests were undertaken to find a combination of equally innocuous buffers. Fowl spermatozoa retained high motility in isotonic diluents B and F containing phosphate buffer and these were used as standards for the comparison of other buffers. All

## TABLE 3

EFFECT OF pH, TONICITY, SODIUM CHLORIDE, AND GLUCOSE CONCENTRATIONS ON THE MOTILITY INDICES OF FOWL SPERMATOZOA

| Diluents | Tonicity | pH = 5·7±0·1 | | | | | | pH = 7·1±0·1 | | | | | | pH = 8·8±0·2 | | | | | | pH = 9·5±0·2 | | | | | | Grand Totals |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ejaculate No. | | | | | Total | Ejaculate No. | | | | | Total | Ejaculate No. | | | | | Total | Ejaculate No. | | | | | Total | |
| | | 1 | 2 | 3 | 4 | 5 | | 1 | 2 | 3 | 4 | 5 | | 1 | 2 | 3 | 4 | 5 | | 1 | 2 | 3 | 4 | 5 | | |
| A | 50 | 1 | 5 | 0 | 5 | 2 | 13 | 31 | 51 | 22 | 33 | 41 | 178 | 18 | 38 | 4 | 35 | 19 | 114 | 0 | 0 | 0 | 0 | 0 | 0 | 305 |
| B | 100 | 12 | 8 | 1 | 15 | 7 | 43 | 61 | 62 | 28 | 61 | 77 | 289 | 8 | 6 | 1 | 9 | 9 | 33 | 1 | 0 | 0 | 2 | 2 | 5 | 370 |
| C | 150 | 18 | 16 | 9 | 34 | 22 | 99 | 67 | 45 | 32 | 71 | 76 | 291 | 3 | 3 | 2 | 9 | 6 | 23 | 2 | 1 | 8 | 2 | 1 | 13 | 426 |
| D | 200 | 3 | 7 | 2 | 26 | 10 | 48 | 50 | 0 | 13 | 67 | 55 | 185 | 3 | 3 | 1 | 12 | 2 | 21 | 0 | 5 | 0 | 1 | 1 | 7 | 261 |
| E | 50 | 0 | 0 | 0 | 1 | 0 | 1 | 38 | 53 | 34 | 54 | 62 | 241 | 2 | 11 | 0 | 3 | 0 | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 258 |
| F | 100 | 12 | 15 | 1 | 25 | 11 | 64 | 65 | 55 | 42 | 67 | 70 | 299 | 37 | 51 | 12 | 60 | 43 | 203 | 1 | 4 | 0 | 2 | 5 | 12 | 578 |
| G | 150 | 5 | 24 | 2 | 32 | 4 | 67 | 70 | 58 | 27 | 73 | 75 | 303 | 63 | 27 | 15 | 18 | 42 | 165 | 1 | 6 | 1 | 4 | 3 | 15 | 550 |
| H | 200 | 0 | 4 | 0 | 4 | 1 | 9 | 18 | 3 | 3 | 68 | 9 | 101 | 0 | 2 | 0 | 7 | 3 | 12 | 0 | 4 | 0 | 1 | 2 | 7 | 129 |
| Totals | | 51 | 79 | 15 | 142 | 57 | 344 | 400 | 327 | 201 | 494 | 465 | 1887 | 134 | 141 | 35 | 153 | 124 | 587 | 5 | 20 | 9 | 11 | 14 | 59 | 2877 |

comparisons were made at a common overlapping pH as close as possible to neutrality. The citric acid–phosphate $v$. phosphate comparison was made at pH 6·1 and the carbonate–bicarbonate $v$. phosphate comparisons at pH 8·4. Five ejaculates were used in each test and motility was scored at $\frac{1}{2}$, $1\frac{1}{2}$, 3, and 5 hr from the start of the test.

The results are shown with summary analyses of variance in Table 2. The motility score in carbonate–bicarbonate and citric acid–phosphate buffer did not differ significantly from that in phosphate and these three buffers were used in subsequent experiments.

TABLE 4

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA
OF TABLE 3

| Source of Variation | Degrees of Freedom | Variance Ratios |
|---|---|---|
| Between ejaculates | 4 | 16** |
| Between pH levels | 3 | 210** |
| Between diluents | 7 | 15** |
| Interactions: | | |
| Ejaculate × pH | 12 | 4·5** |
| Ejaculate × diluent | 28 | 1·1 |
| Diluent × pH | 21 | 5·6** |
| Residual | 84 | 77 |

$**P < 0.01$.

### (b) Systematic Studies

The motility of fowl spermatozoa was studied at five pH levels (nominally 4·0, 5·5, 7·0, 8·5, and 10·0). At each pH level, the eight diluents of Table 1 were used, so that at four tonicity levels, the effect of substituting sodium chloride for part of the glucose could be examined. All experiments were repeated five times, all ejaculates (usually pooled specimens) being partitioned between tubes representing all possible combinations of the factors investigated.

The results are set out in Table 3, omitting the results for pH 4·0 as the spermatozoa were immotile in all diluents. Fowl spermatozoa had an optimum pH at about 7 and, although there was a rapid fall off in motility on either side of the optimum pH, at least partial motility was observed from pH 5·7 to 9·5. The pH of fowl semen lies close to the optimum pH for motility, thus the mean of 18 ejaculates was 7·6 (S.D.=0·42; range 8·1–6·9). The summary of the overall analysis of variance (Table 4) shows highly significant differences between pH levels and highly significant interactions. The results have therefore been analysed separately at each pH (Table 6) according to the scheme set out in Table 5.

The single significant difference in hypertonic diluents D and H at pH 5·7 may be fortuitous, as the greater part of the difference was contributed by ejaculate 4. All ejaculates did, however, show a slight advantage of diluent D over H.

At pH 7·1 fowl spermatozoa showed considerable tolerance to changes in tonicity, the optimum being between 100 and 150.   At the extreme tonicities of 50

TABLE 5

SCHEME ADOPTED FOR ANALYSIS OF RESULTS IN TABLE 6

| Source of Variation | Factorial Coefficients for Diluents: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H |
| Part replacement of glucose by NaCl, tonicity 50 | +1 | 0 | 0 | 0 | −1 | 0 | 0 | 0 |
| Part replacement of glucose by NaCl, tonicity 100 | 0 | +1 | 0 | 0 | 0 | −1 | 0 | 0 |
| Part replacement of glucose by NaCl, tonicity 150 | 0 | 0 | +1 | 0 | 0 | 0 | −1 | 0 |
| Part replacement of glucose by NaCl, tonicity 200 | 0 | 0 | 0 | +1 | 0 | 0 | 0 | −1 |
| Curve fitting: | | | | | | | | |
| Linear regression | −3 | −1 | +1 | +3 | −3 | −1 | +1 | +3 |
| Simple curvature | +1 | −1 | −1 | +1 | +1 | −1 | −1 | +1 |
| Double curvature | −1 | +3 | −3 | +1 | −1 | +3 | −3 | +1 |

and 200, they tended to adapt themselves to their unfavourable environment and, although their motility was very depressed at the start of the tests, it increased so that

TABLE 6

SUMMARY OF THE ANALYSES OF VARIANCE AT SEPARATE pH LEVELS FOR THE DATA OF TABLE 3

| Source of Variation | Degrees of Freedom | Variance Ratios at pH: | | |
|---|---|---|---|---|
| | | 5·7 | 7·1 | 8·8 |
| Between diluents | | | | |
| Glucose × NaCl (tonicity 50) | 1 | 0·5 | 2·1 | 9·5** |
| Glucose × NaCl (tonicity 100) | 1 | 1·5 | 0·1 | 28·6** |
| Glucose × NaCl (tonicity 150) | 1 | 3·5 | 0·1 | 20·0** |
| Glucose × NaCl (tonicity 200) | 1 | 5·2* | 3·8 | 0·1 |
| Curve fitting | 3 | 44·4** | 34·8** | 22·7** |
| Between ejaculates | 4 | 9·5** | 9·2** | 2·8** |
| Residual | 28 | 29 | 185 | 101 |

*$P < 0.05$.          **$P < 0.01$.

the $1\frac{1}{2}$-hr scores were higher than the $\frac{1}{2}$-hr scores.   Thus when the $\frac{1}{2}$- and $1\frac{1}{2}$-hr scores for diluents A, D, E, and H and B, C, F, and G (Table 7) are subjected to analyses

of variance, diluents A, D, E, and H showed a significant rise ($P<0.01$), and diluents B, C, F, and G a significant fall ($P<0.05$), in motility. These effects are illustrated in Figure 1.

TABLE 7

SUM ($\times 4$) OF THE MOTILITY SCORES FOR FIVE FOWL EJACULATES AT $\frac{1}{2}$ AND $1\frac{1}{2}$ HR RESPECTIVELY IN DILUENTS AT pH 7·1

Individual ejaculate scores have been omitted from the table but were used in the analysis of variance

| Unfavourable Conditions | | | | | Favourable Conditions | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Time (hr) | | | | | Time (hr) | | |
| Diluent | Tonicity | | | Total | Diluent | Tonicity | | | Total |
| | | $\frac{1}{2}$ | $1\frac{1}{2}$ | | | | $\frac{1}{2}$ | $1\frac{1}{2}$ | |
| A | 50 | 34 | 43 | 77 | B | 100 | 73 | 68 | 141 |
| D | 200 | 27 | 40 | 67 | C | 150 | 69 | 68 | 137 |
| E | 50 | 44 | 59 | 103 | F | 100 | 73 | 69 | 142 |
| H | 200 | 17 | 18 | 35 | G | 150 | 74 | 68 | 142 |
| Totals | | 122 | 160 | 282 | | | 289 | 273 | 562 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Variance Ratios | |
|---|---|---|---|
| | | Unfavourable Conditions | Favourable Conditions |
| Between diluents | 3 | 32·9** | 0·0 |
| Between times | 1 | 15·0** | 5·7* |
| Between ejaculates | 4 | 19·6** | 13·3** |
| Interactions: | | | |
| Diluent $\times$ time | 3 | 1·7 | 0·0 |
| Diluent $\times$ ejaculate | 12 | 10·8** | 1·1 |
| Time $\times$ ejaculate | 4 | 3·3* | 2·0 |
| Residual | 12 | *2·4* | *1·5* |

*$P<0.05$.    **$P<0.01$.

At pH 8·8 there were highly significant differences between glucose and chloride diluents at tonicities of 50, 100, and 150 (Table 6). In all cases, the diluents containing little or no chloride were superior to the diluents of high chloride content. If diluents A and B, containing equal amounts of glucose, are compared it will be seen that the chloride-containing diluent B is less favourable than the chloride-free diluent A even though the tonicity of diluent B is more conducive to survival. This points to a detrimental effect of sodium chloride on the survival

of spermatozoa at this pH. Table 8 gives the results of confirmatory tests on four ejaculates using the carbonate–bicarbonate buffer at pH 8·6. The sodium chloride percentage was varied logarithmically from 0 to 0·17 at tonicity 50 and from 0 to 0·4 at tonicity 100, the tonicity in each diluent being kept constant by adjusting the glucose level. The summary analyses of variance (Table 8) showed in each case a significant linear fall in motility with increasing chloride concentration.

TABLE 8

EFFECT OF SODIUM CHLORIDE CONCENTRATION ON THE MOTILITY INDICES OF FOUR FOWL EJACULATES AT pH 8·6

| | Tonicity 50 | | | | | | Tonicity 100 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NaCl (%) | Ejaculate No. | | | | Total | NaCl (%) | Ejaculate No. | | | | Total |
| | 1 | 2 | 3 | 4 | | | 1 | 2 | 3 | 4 | |
| 0·00 | 67 | 61 | 69 | 70 | 267 | 0·0 | 80 | 80 | 80 | 72 | 312 |
| 0·04 | 68 | 59 | 67 | 45 | 239 | 0·1 | 80 | 80 | 78 | 61 | 299 |
| 0·08 | 69 | 33 | 63 | 34 | 199 | 0·2 | 73 | 77 | 72 | 66 | 288 |
| 0·17 | 19 | 10 | 38 | 22 | 89 | 0·4 | 75 | 30 | 22 | 39 | 166 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Variance Ratios | |
|---|---|---|---|
| | | Tonicity 50 | Tonicity 100 |
| Between ejaculates | 3 | 3·6* | 1·6 |
| Between diluents: | 3 | 16·2** | 7·8** |
|    Linear | 1 | 43·8** | 17·1** |
|    Quadratic | 1 | 4·5 | 5·0 |
|    Cubic | 1 | 0·5 | 1·1 |
| Residual | 9 | *94* | *147* |

*$P < 0.05$.          **$P < 0.01$.

## IV. DISCUSSION

Fowl spermatozoa are motile over a similar pH range (5–10) to that of the other species studied, viz. the rabbit, ram, bull, and human (Emmens 1947, 1948; Blackshaw and Emmens 1951) and have an optimal pH for motility of about 7. At high pH levels fowl spermatozoa tend to become susceptible to the harmful effect of increasing sodium chloride concentration and its replacement by glucose is beneficial. This effect has been previously observed with mammalian spermatozoa in hypertonic media (Emmens 1948; Blackshaw and Emmens 1951). The results reported here, however, are much more striking, and suggest that the phenomenon is a general characteristic of vertebrate spermatozoa. There is no obvious explanation as to why

sodium chloride should be particularly harmful at alkaline pH values; the beneficial action of replacing chloride by glucose in this and other work is, however, due to the maintenance of viability throughout the experimental period rather than to an initial increase in motility.

Fowl spermatozoa are particularly resistant to hypertonicity. They resemble ram, bull, and human spermatozoa in their response to changes in tonicity (Blackshaw and Emmens 1951) and do not show the interaction between pH and tonicity characteristic of rabbit spermatozoa (Emmens 1948).
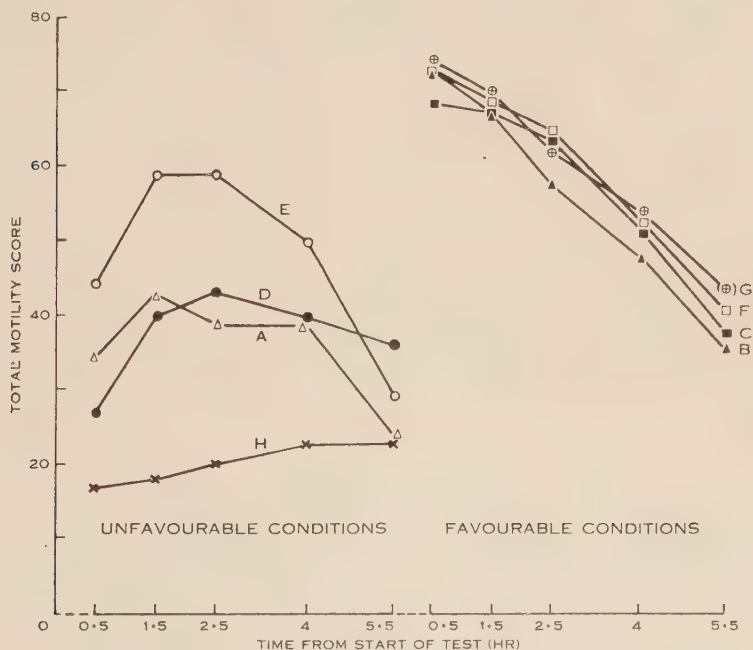


Fig. 1.—The motility scores (sum × 4) of five fowl ejaculates at pH 7·1, in diluents A–H (see Table 1), showing the adaption of fowl spermatozoa to unfavourable conditions during the first 1½ hr of the experiment.

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# THE EFFECTS OF DAY LENGTH ON THE COAT-SHEDDING CYCLES, BODY WEIGHT, AND REPRODUCTION OF THE FERRET

By Nancy E. Harvey* and W. V. Macfarlane*

*Summary*

The effect of light on the coat of the ferret has been measured during the annual cycle of seasons. Two groups of ferrets were kept in the same environment except for the daily duration of light, which comprised the normal seasonal day length of Brisbane for the control group, and a complete and exaggerated reversal of seasonal light trend for the experimental group.

The growth of hair was shown to be controlled by day length. In both control and experimental groups, as the daily light decreased, there was an extensive shedding of hair, stimulation of new hair growth, and a subsequent increase in coat thickness. As days became shorter, body weight increased significantly, sebum secretion declined, and both males and females became reproductively inactive.

When day length increased, on the other hand, hair growth ceased and minor shedding of some of the fine hair decreased the density of the coat. This resulted in a relatively coarse coat, which became very greasy with sebaceous secretion. There was a significant fall in body weight and the ferrets rapidly assumed reproductive activity as days lengthened, irrespective of ambient temperature.

The effects of pregnancy and other factors which may be associated with these changes are discussed.

## I. Introduction

That light is an important factor in regulating the breeding season of ferrets has frequently been demonstrated since the initial studies made by Bissonnette (1932). He showed that electric light added after dusk caused the onset of oestrus in the ferret during the non-breeding season of the year, from September to March (northern hemisphere) or from March to September (southern hemisphere).

Later it was reported that changes in the pelts of ferrets and fitch (Bissonnette 1935) and mink (Bissonnette and Wilson 1939) were conditioned by changes in the duration of daily exposures to light irrespective of the environmental temperature and colour of surroundings. A more detailed account is given by Bissonnette and Bailey (1944) of studies on the weasel, an animal closely related to the ferret. They found that reduction of the daily periods of light induced moulting and growth of new fur, together with a colour change from brown to white (Bonaparte weasels) or from dark brown to light brown (New York weasels). Increase of the daily light periods caused moulting and a change to dark brown in both species. It was also found that a latent or adjustment period intervened between the time of increase of light ration and the shedding and regrowth of hair. In the weasel the latency appeared to be about 3 months. It was noted by Hammond Jr. (1953) that mink laid down thick subcutaneous fat during the period of decreasing light but the deposition was not measured. This adaptation to cold appeared to be unaffected by temperature since it occurred when day temperatures were over 90°F if the photoperiod were reversed.

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Photostimulation passes through the eyes, as has been shown by Bissonnette, and the fact that hypophysectomy abolishes the cyclic moulting and regrowth of hair in ferrets (Bissonnette 1935) indicates that this gland is involved in the reaction. Recent studies by Donovan and Harris (1954) have demonstrated that the effect of light on oestrus in the female ferret depends upon the integrity of the hypophysial portal blood vessels. It seems likely, therefore, that the seasonal light change acts as a stimulus which is translated into a seasonal release of hormones from the anterior pituitary, and that this controls the breeding cycle, and probably the hair cycle also.

Cattle shed their coats under the influence of changes of day length (Yeates 1955) and the ferret has been studied as a laboratory animal in order to investigate the control of the shedding process.

## II. Experimental Methods

Five male and five female adult ferrets, including four albino ferrets, were kept in large wire cages in a room open to natural daylight. They were therefore exposed to the normal light duration for the latitude of Brisbane, 27°30′S. These formed the control group.
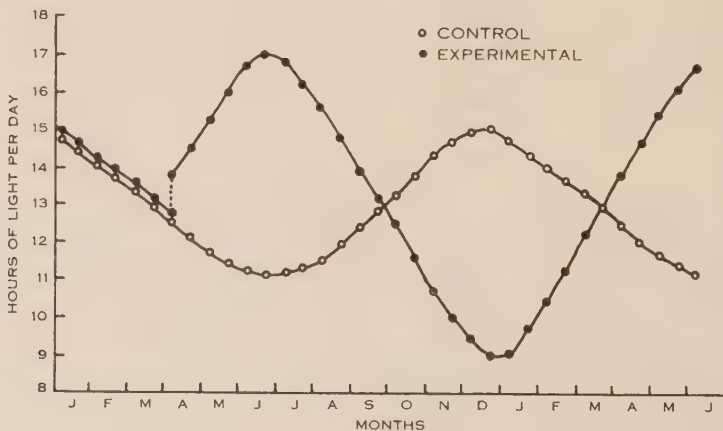


Fig. 1.—Lighting conditions to which the control and experimental groups were subjected.

A similar group of ferrets was placed in a box 6 by 4 by 3 ft in which the duration of light could be decreased by blackout arrangements, or increased by switching on four 100-W electric lights in the early morning and evening. A pattern of light duration similar to that used by Yeates (1955) was adopted for the experimental group. From Figure 1 it is seen that while the control animals were experiencing their shortest day in June, the experimental ferrets through the addition of artificial light were experiencing their longest days. The summer-to-winter light changes in the experimental group were exaggerated beyond the normal range for Brisbane, so that they amounted to 17 hr of light in midwinter and 9 in midsummer. This was done to emphasize any effects which light might have. Apart from reversal of the light trend for the experimental group, the environments and treatments of both groups were the same. The animals were exposed to the normal temperature fluctuations for Brisbane's

climate, ranging from a winter mean monthly minimum of 49°F, to a summer mean monthly maximum of 86°F. To prevent any excessive rise in temperature within the light box, fresh cool air was circulated and maximum–minimum thermometers showed that the temperatures of the control room and light box did not differ by more than 3°F.

A diet of raw meat, bread, and water supplemented with bran mash, salt, and vitamins was provided *ad libitum*. Satisfactory weight records and freedom from disease indicated that there was no nutritional deficiency.

TABLE 1

CHANGE IN AVERAGE LENGTH OF COARSE AND FINE HAIR (CALCULATED FROM THE LENGTHS OF ABOUT 20 HAIRS FROM CLIPPINGS OF FOUR CONTROL AND FIVE EXPERIMENTAL MALES) AFTER SHEDDING INDUCED BY DECREASING DAILY PHOTOPERIOD

|  | Control | | Experimental | |
|---|---|---|---|---|
|  | Coarse Hair | Fine Hair | Coarse Hair | Fine Hair |
| Average maximum length (mm) before shedding period | $48 \pm 5$ | $28 \pm 5$ | $46 \pm 5$ | $33 \pm 5$ |
| Average maximum length (mm) after 4–6 weeks of major coat-shedding period | $33 \pm 3$ | $21 \pm 1$ | $31 \pm 3$ | $23 \pm 3$ |
| Significance of difference | $P < 0.001$ | $P < 0.05$ | $P < 0.001$ | $P < 0.01$ |

During 12 months, observations were made on the reproductive state of the animals, and weekly records of weight were kept. In particular, measurements were made on the coat. The shedding tendency was estimated by the amount of hair which could be pulled from the coat by gently plucking. A scoring system was used by which increasing degrees of shedding were represented by numbers up to 4. Hair density was calculated by shaving a measured area of skin and weighing the hair so removed. This sample of hair was analysed by measuring the lengths and diameters of about 200 individual hairs from each sample. The growth rate of hair was measured directly on the shaved areas as millimeters increment per week. The activity of the sebaceous glands was assessed roughly by the greasiness of the coat.

During the course of the year's experiment two animals died from causes unknown. The results given below are therefore averages from four male and five female control ferrets, and from five male and four female experimental ferrets.

### III. RESULTS

#### (a) Hair

(i) *Type of Hair*

*Diameter.*—Two distinct types of hair occur in the coat of ferrets—coarse hairs with a diameter of $95 \pm 20$ $\mu$ and fine hairs with a diameter of $23 \pm 9$ $\mu$. The changing proportions of fine and coarse hairs of male ferrets are shown in Figure 2. There was relatively more coarse hair in the long light period, while the fine hair correspondingly

decreased. In the short light period there was a decrease in the relative amount of coarse hair and an increase of fine hair. The differences within each group and between the two groups are significant at the $P < 0.001$ level.

Some of the females were allowed to mate, and the ensuing pregnancy or pseudo-pregnancy had pronounced effects on the coat. The females were therefore excluded from the analysis of the effect of light on the coarseness of the coat. The effects of pregnancy will be discussed later on in more detail.
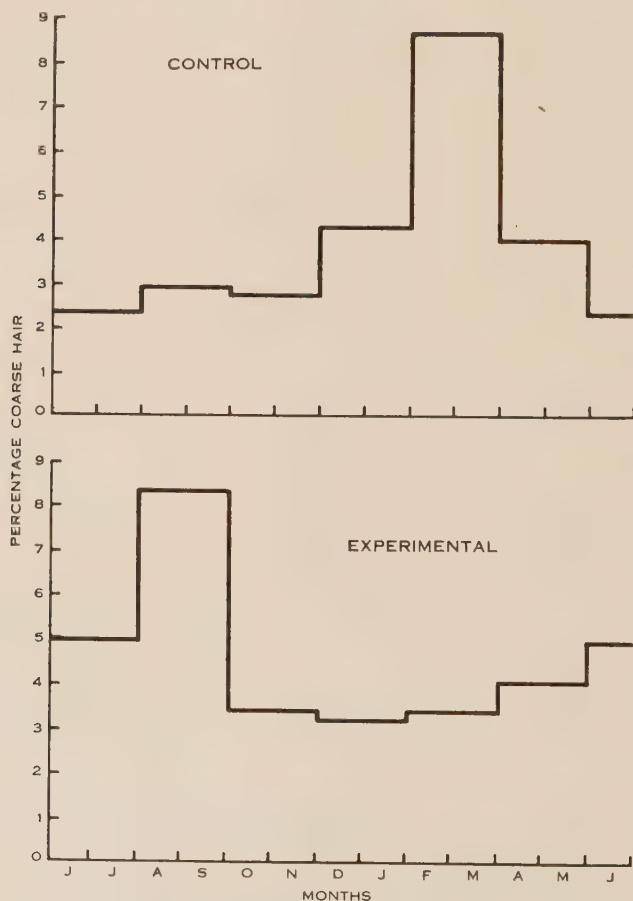


Fig. 2.—Percentage of coarse hairs (diameter $\simeq 95\,\mu$) in coats of male ferrets.

*Length*.—The changes in hair length were less obvious. The maximum lengths of hair in midsummer and midwinter were not significantly different for either group of ferrets. There was, however, a significant decrease in average coat length at the beginning of the major shedding period (Table 1). Since rapid growth also occurred in this period, the hair quickly became thick and long again within 4–6 weeks.

*Greasiness*.—The coat appeared oily and the hairs became adherent to each other as the light increased, and the coat was greasy to touch in contrast with the soft, non-greasy coat of the short light season.

*Pigmentation.*—Pigmentation, when present, occurred only in the distal portion of the coase hairs, which varied in colour from light brown to black. Fine hairs of all the ferrets remained unpigmented, although sebum secretion tended to turn them yellowish in the "summer" season.
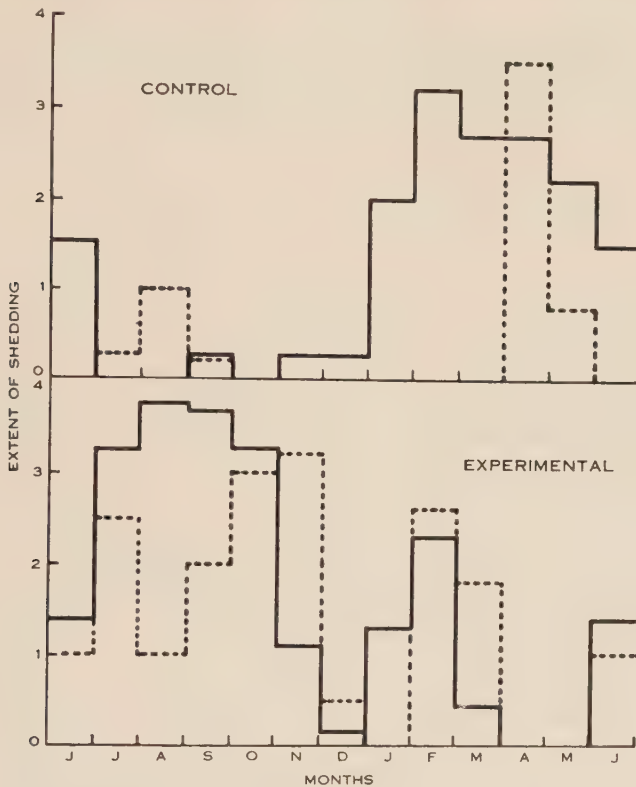


Fig. 3.—Extent of shedding in male (———) and female (- - - -) ferrets. Increasing degrees of shedding are represented by figures up to a maximum of 4.

## (ii) *Shedding*

For each group of ferrets there were two distinct moults during the year—a major shed during the decreasing light period and a minor shed during the increasing light period. The major shed for the control males lasted from January to June with a peak in February, so that the response to the change in light trend occurred after 1 month of shortening day length. The major shed for females, excluding the two which were pregnant at this time, was much shorter, lasting for only 2 months (April and May) with a peak in April. The latent period for females was about 3 months.

The minor shed for the control males occurred from September to December, 3 months after the light began to increase. From July to September there was minor shedding among the females after a latent period of 1 month.

The experimental animals showed the same shedding pattern as the controls, except that it was 6 months out of phase. The major shed lasted from June to November with a peak in August-September for the males and in October-November

for the females. The latent period for the males, therefore, was less than 1 month, whereas for the females it was about 3 months.

The lesser shed in the males occurred from January to March and from February to March in the females. Shedding reached a peak in February for both groups, i.e. after 1 month's latency.

The major shed involved a loss of the entire coat of old hair in a wavelike fashion, starting at the head and proceeding in roughly transverse bands towards the tail.
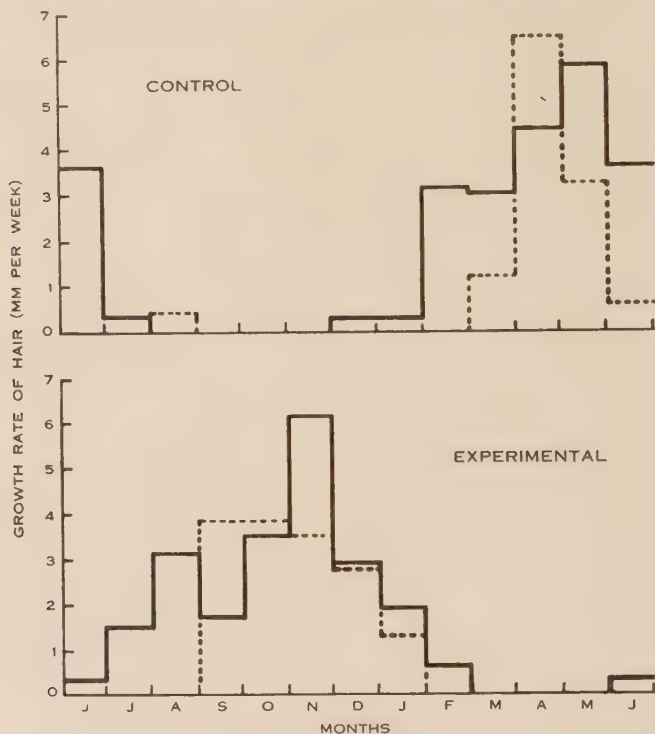
Fig. 4.—Growth rate of hair (mm per week) of male (———) and female (– – – –) ferrets.

New hair, already partly grown, took the place of the old coat, and although the animals were never actually bare at any time, the coat became short during this moulting process. The process was particularly clear in the white ferrets whose "summer light" coat was coarse, and yellow with grease. As shedding proceeded, the yellow hair was replaced, band after band, by soft white hair. The minor shed appeared to involve only the loss of some of the underfur, while the thicker hairs became coated with sebum. The extent of shedding, averaged over the groups, is shown in Figure 3. The prolific shedding which followed in the two pregnant control animals is not included in the histogram.

(iii) *Growth Rate of Hair*

The average rate of growth of hair for each group of animals is shown in Figure 4. There was virtually no growth during the period of increasing daily light so the

clipped patches remained bare for nearly 6 months of the year. Growth was stimulated within 4 weeks of decreasing the daily light and the greatest rate of growth (6 mm/week) was reached 4 or 5 months later. The graph for the control females does not include the rapid growth of hair which occurred in two ferrets in December and early January as a result of pregnancy.

The hair reached its maximum length (Table 1) before it was fully pigmented, so that clipped patches were easy to identify for measuring purposes. Full pigmentation of the coarse hair occurred 3 or 4 weeks after the hairs had grown to their maximum length.
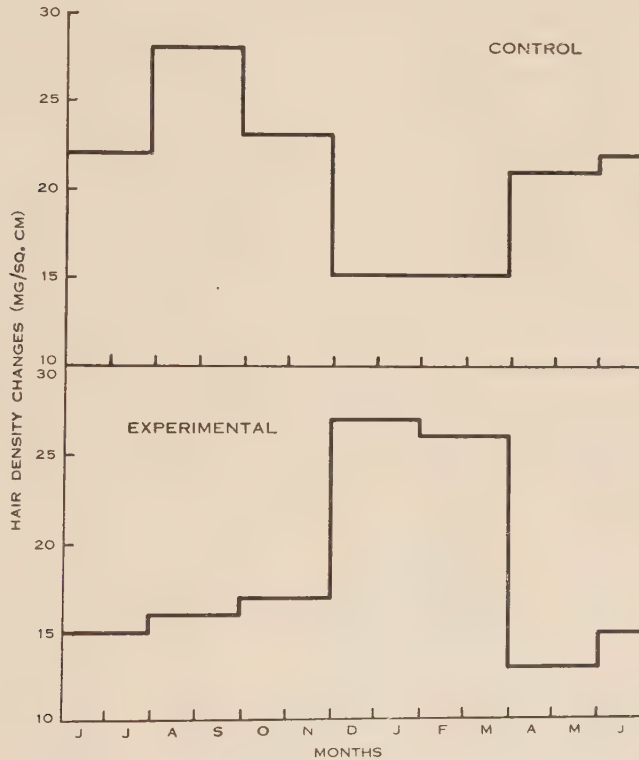


Fig. 5.—Changes in density of hair (mg/sq. cm.) among male ferrets.

(iv) *Density of Hair*

Changes in coat density with the seasonal light changes were analysed on the male ferrets of each group (Fig. 5). In the long day period the coat became less dense as the minor shedding process brought about loss of fine underfur. After a latent period of about 2 months, shortening day length was associated with an increase in coat density, which reached its maximum 2 or 3 weeks after the shortest day. The difference in coat density between the groups for "summer" and "winter" was highly significant ($P<0.001$).

The females are excluded from this analysis again owing to the effects of pregnancy.

## (b) Breeding Season

The breeding season of both male and female ferrets was recorded in terms of the size of the testes or vulva, and its incidence is represented in Figure 6. The histograms show that the females were in full oestrus for about 6 months of the year and completely anoestrous for the other 6 months. The sexual activity of the males, on the other hand, reached a peak for only about 4 months, but it was sustained at a much reduced level during most of the rest of the year.
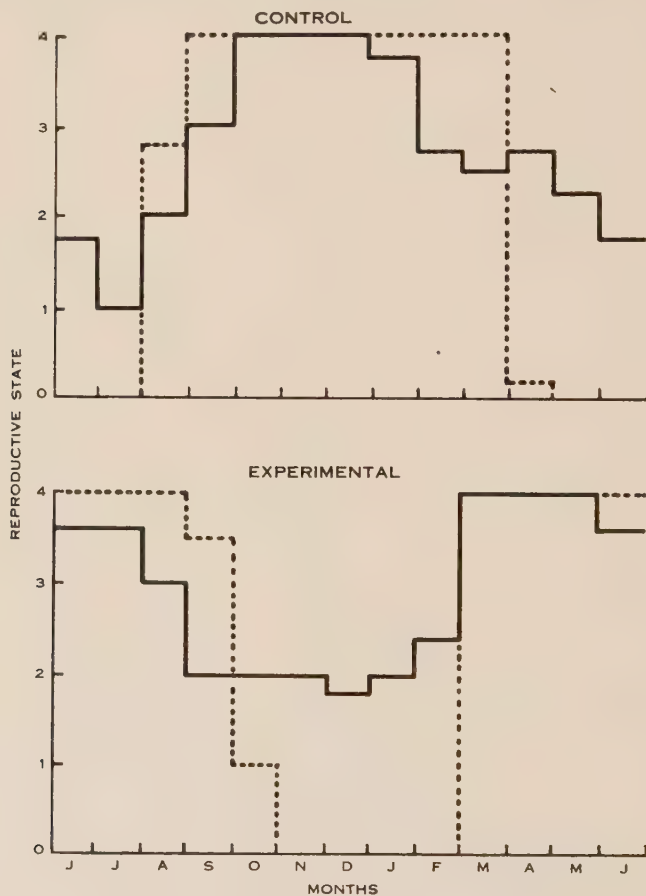


Fig. 6.—Reproductive state of male (———) and female (– – – –) ferrets. The size of the testes or vulval turgor is indicated by an arbitrary scale of 0 (small) to 4 (large, active).

The relationship of breeding cycles and light changes is in accord with the many previous experiments reported since 1932. It is presented here to show its correlation with the hair cycle.

## (c) Body Weight

Weight gain or loss in fully grown ferrets was found to be a light-controlled seasonal phenomenon (Figs. 7 and 8). As the daily light period increased there was a gradual loss of weight amounting to 22–28 per cent. of the initial weight. The weight cycle was reversed by reduction of day length but it was independent of ambient tem-

perature. Hence the animals were lighter in weight during long days and heavier during short days. The differences between the summer and winter values (January and July) in each curve are highly significant ($P < 0.001$), as are the differences between the two January and two July peaks (controls and experimentals).
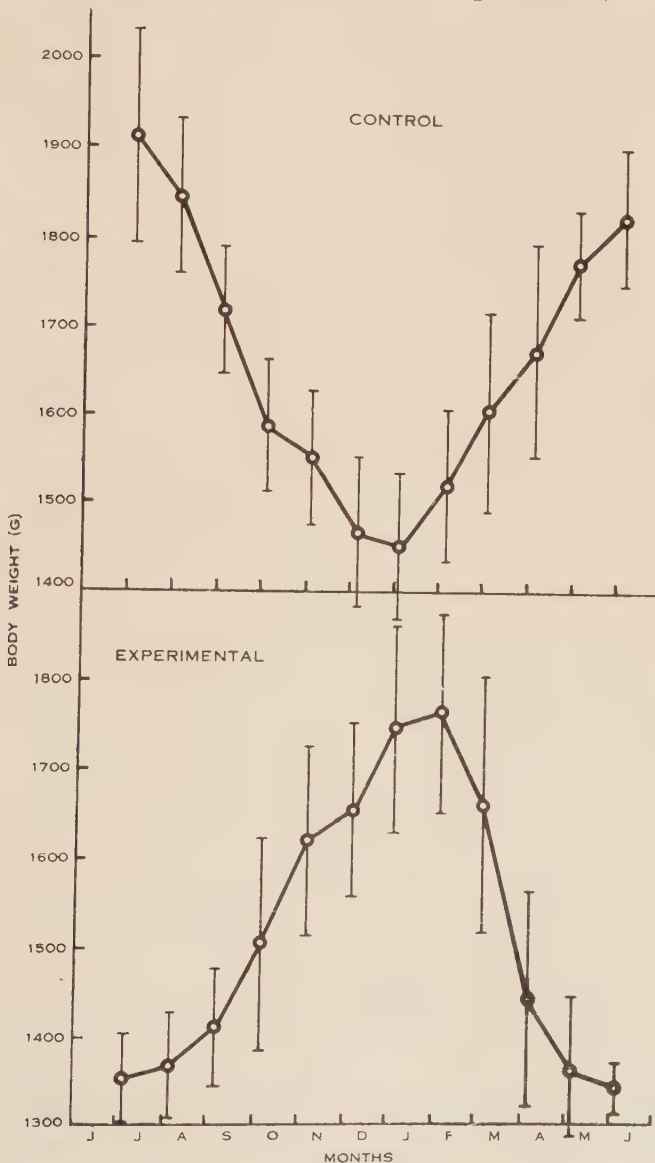


Fig. 7.—Body weight (in grams) of fully grown male ferrets, showing the standard deviation for each month.

## (d) Pregnancy

The effect of pregnancy on the coat of the ferret was similar to that produced by reducing the daily photoperiod. About 2 weeks after mating, the vulva subsided,

indicating an anoestrous state, and at this time coat shedding commenced. Hair growth was rapid, and the coat became sleek and free from excess grease, despite the season of the year. Parturition occurred 6 weeks after mating. In the absence of
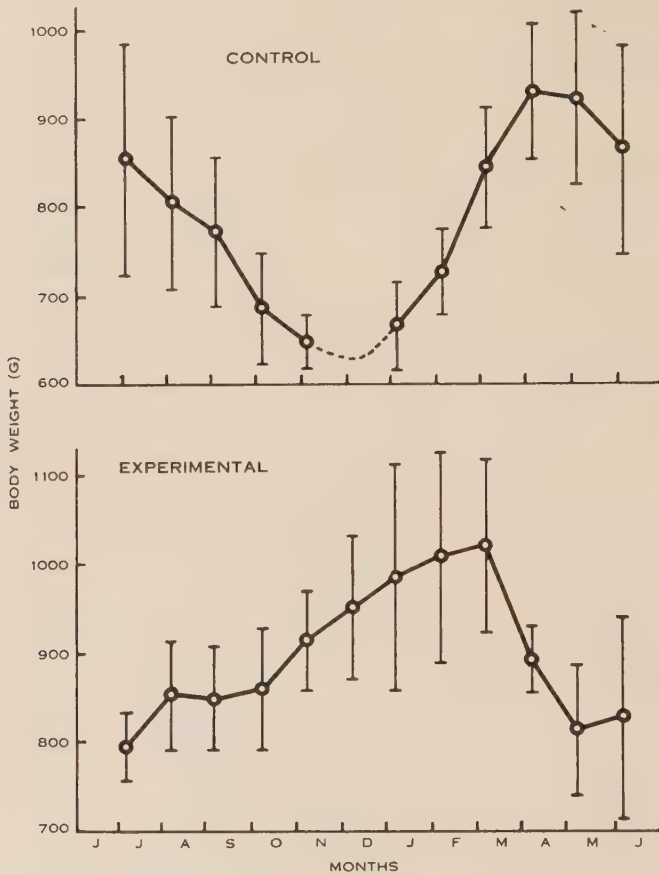


Fig. 8.—Body weight (in grams) of adult female ferrets showing the standard deviation for each month.

lactation, the vulva swelled to the oestrous condition again within 1 week. Shedding, however, continued about 4 weeks after parturition, and hair growth was rapid during this post-partum period. Eventually this activity ceased, and the animals returned to the condition typical of the increasing daylight period. When lactation followed parturition, there was a longer post-partum period (about 6 or 7 weeks), during which there was no vulval turgor, while hair growth and shedding continued.

### (e) Sequence of Events

From a composite diagram representing the average behaviour of four male control ferrets (Fig. 9) it appears that slight shedding during the increasing photo-period ("spring") caused the density of hair to fall, since there was no compensatory

hair growth at this time. After the change over to a decreasing photoperiod, although growth of hair was stimulated, the effect was counteracted by massive shedding, so that for about 2 months the density of hair remained low. Eventually the hair growth
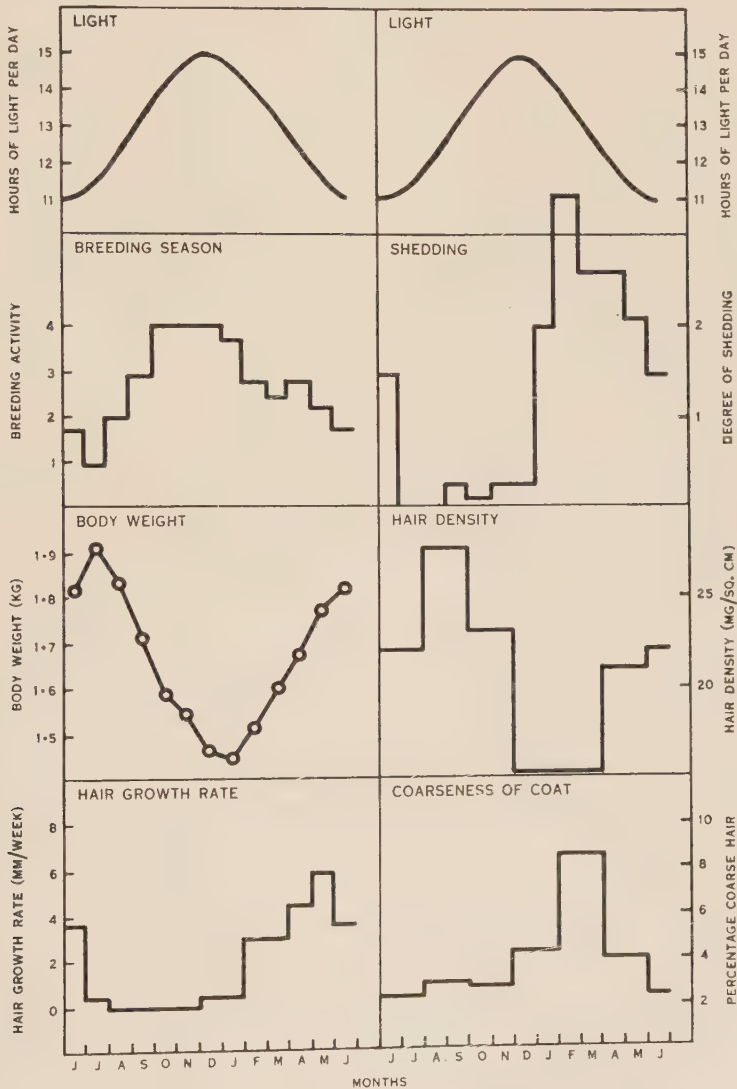


Fig. 9.—Correlation of light changes with breeding season, body weight, hair growth, shedding, hair density, and coarseness of coat of the control male ferrets.

rate exceeded the shedding rate so that the coat became thicker by "winter". It is apparent, too, that the minor shed involved mainly fine hair since the relative amount of coarse hair in the coat increased. This coarse hair was lost in the next (major) shedding process.

The curves for the time course of breeding season and body weight were almost exactly 180° out of phase. Body weight increased only as breeding activity declined, and growth of hair occurred only as body weight increased.

## IV. DISCUSSION

The coat of the ferret consists of two distinct types of hair—coarse overhair and fine woolly underfur. In the normal summer, i.e. when daylight is longest, the coarse hairs number about 8 per cent. of the coat, and the fine hair is relatively short. The coat is sparse and greasy with sebum so that air circulates into the coat, which offers little insulation. There is no new growth of hair, and the shedding, if any, is slight and comprises fine underfur only. As day length decreases, hair growth is quickly stimulated and the major shedding process involving the entire coat of summer hair begins. This keeps the density of the coat low until the growth rate of hair exceeds the shedding rate. Like mouse hair patterns (Nay and Fraser 1954), the hair follicles of the ferret appear to become activated in waves passing towards the tail. Shedding and hair growth occur first on the head, and then proceed gradually in bands from the head, so that the tail is the last part to lose the old coat and the last to grow the new one. The new coat has only 2 or 3 per cent. of coarse hairs and, by midwinter, the hair has grown to its maximum length. The sebum secretion is reduced, and the normal winter coat is, therefore, a soft, thick insulator.

If there is a hormonal stimulus to growth or shedding, it must be assumed that there are many different types of follicle with different latencies and degrees of response. Growth of some hairs occurs at the same time as loss of other hairs. This suggests a life cycle of individual follicles in which the endocrine background may stop follicular proliferation of hair cells in one, while encouraging growth in another. There remains the possibility of local action, in which a shedding follicle could release a stimulating substance to increase the growth of neighbouring follicles.

The breeding season is brought on by the increasing photoperiod. The males respond to the change in light trend within 1 month, while the females have a latent period of 1–2 months before a change in vulval turgor is produced.

In the adult ferret there is a loss of body weight during the period of increasing day length, and a gain of weight during the next 6 months of decreasing light. The exact nature of this gain and loss—protein, fat, water, or otherwise, has not been investigated, but its inverse relation to breeding season suggests that weight loss is due to greater activity during the reproductive period.

The complete reversal of these phenomena by 6 months reversal of day length, other factors of the environment being the same, is good evidence that light determines coat changes as well as the complex adaptation of reproduction. The breeding cycle and hair-shedding cycle may be causally linked, and this association has a parallel in the effects of pregnancy on hair growth. It is possible that light may influence hair growth through a seasonal release of gonadal hormones. For instance, whenever the female ferret becomes anoestrous—whether due to decreasing light or to pregnancy— there is stimulation of hair growth and shedding takes place. In the male, too, there is no hair growth until seasonal breeding activity is reduced. This indicates that oestrogens should inhibit, and progesterone encourage, both growth and shedding.

It has, in fact, been found in this Laboratory that growth and shedding of hair follow progesterone treatment during oestrus, though treatment with oestradiol has so far been ineffective as an inhibitor of hair growth during anoestrus.

It has been known for some time (Bissonnette 1935) that hypophysectomy abolishes the cyclic hair patterns in ferrets, and it may well be that this is consequent upon the abolition of breeding cycles (Hill and Parkes 1933).

On the other hand, there is also the possibility that hair growth and shedding are influenced by a seasonal release of some other endocrine factor or factors, which are also affected by pregnancy. Sebaceous secretion and the minor ("spring") shedding occur together. It is possible that the same factor could induce both changes. Evidence has been found for a pituitary tropic factor acting on sebaceous glands in the rat, which, although contained in some gonadotrophic fractions, varies independently of gonadotrophic activity (Lasher, Lorincz, and Rothman 1955). It seems possible that this hormone or others like it may influence the skin and hair cycles. Experiments are now being carried out to investigate this problem.

## V. Acknowledgments

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# STUDIES OF SHEEP MOSAIC FOR FLEECE TYPE

## I. PATTERNS AND ORIGIN OF MOSAICISM

### By A. S. Fraser* and B. F. Short†

*Summary*

Sheep which are mosaic for fleece type are described with emphasis on the patterns of distribution of areas of the mutant fleece type over the body. Probable causes of the underlying mosaicism of skin tissues are discussed.

## I. INTRODUCTION

Recently we have made a collection of sheep which are mosaic for fleece type. Most of these are Merinos or shortwools (Southdown and Dorset Horn), with fleeces of normal staple length over most of the body but which have patches of longer wool varying in area and position from sheep to sheep. The search for such sheep was stimulated by a description of a Merino fleece mosaic by Lang (1952) and Chapman, Moule, and Richards (1954). It was thought that sheep of this type could be useful in determining the biological control of the component characters of fleece weight and fleece type. The contribution which studies of mosaic fleeces can make to our understanding of fleece growth will be discussed in later papers; in this paper we wish to consider the genetic basis of mosaicism in sheep.

Mosaic individuals are those in which a normally monotypic cell lineage is split into two or more distinct types and it is pertinent to distinguish two types of mosaicism according to the way in which the mosaicism may have come about. These are (i) "developmental" mosaics in which the phenotypic differences in the cell lineage are not paralleled by genetic differences, and (ii) "genetic" mosaics in which a genetic mosaicism underlies the phenotypic one.

The first type includes individuals with broken colour patterns such as "English" rabbits, spotted mice, roan cattle, saddle-back pigs, and so on. Certain lambs which have hairy birthcoats also belong to this category. While it is not absolutely certain that such patterns are unaccompanied by genetic differentiation, there is no evidence that they are. In one instance there is evidence that they are due to something else. This comes from investigations of the white-mottled variegation series in the determination of eye color in *Drosophila melanogaster* where the variegation is due to a translocation or inversion shifting a gene into the heterochromatin. Although it is not possible to show that in such a mosaic the soma is not genetically differentiated, the patterns are nevertheless demonstrably controlled by genotypes which segregate normally in crosses and therefore the controlling factors are present in *all* cells, not just a fraction of them.

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Another class of mosaic, difficult to separate logically from these, appears to be due to a high rate of mutation of a gene or chromosome type. This mosaicism is supposed to be due to *back mutation* because the gene (*a*) has a mosaic expression, and (*b*) has a high mutation rate in the germ line. In these cases the genotype of the germ line is such that the soma to which it gives rise is liable to genetic differentiation by mutation at any stage. It can only be distinguished from the first type of mosaicism if the assumption is made that no genetic differentiation takes place in such cases of spotting or variegation.

Genetic mosaics other than those due to a predisposing genotype may originate as follows: (1) mutation of a gene in an early cell generation; (2) loss of a chromosome or part of a chromosome due to a faulty mitosis at an early cell generation; (3) addition of a chromosome or chromosome set due to a faulty mitosis at an early cell generation; (4) fertilization of the egg by more than one sperm; (5) fusion of two adjacent zygotes; (6) inclusion of cells from another individual (graft mosaics), e.g. erythrocyte mosaics in cattle (Stormont 1954), or sectorial mosaics in pigeons (Hollander 1949).

Conclusive demonstration of the mechanism underlying a genetic mosaic is difficult in organisms other than *Drosophila*, and therefore most analyses have been made in *Drosophila* (Morgan 1914; Sturtevant 1929; Stern 1936; Crew and Lamy 1938; Patterson and Stone 1938). These have shown that genetic mosaics are mostly caused by the elimination of all or a part of a chromosome at an early mitosis. Such analyses as can be made of the mechanisms of genetic mosaicism in sheep are much too limited to produce an unequivocal answer, and therefore this aspect has been given only cursory attention. The main aim here is to describe the essential features of our group of "mosaic" sheep and to discuss those which support the hypothesis that these are true genetic mosaics.

## II. MATERIAL AND METHODS

### (a) Detection of Mosaic Sheep

The search for mosaic fleece types has resulted in the discovery of some 30 mosaics, plus a number of animals with completely abnormal fleeces, presumably due to the action of mutant genes. In all, some 200 sheep have been examined in the field, and 40 of these, regarded as possible mosaics, were transferred to the Sheep Biology Laboratory, C.S.I.R.O., Prospect, N.S.W. Here they were closely shorn and penned individually to allow growth of a new fleece under controlled conditions.

The great majority of sheep inspected had regions of wool which was longer than normal. Only one sheep had regions with wool shorter than normal. The predominance of long-stapled mosaics may be due to observational difficulties since staples which project above the rest of the fleece are more noticeable than staples which lie between a majority of longer, neighbouring staples. Moreover, an increase of fibre length has usually resulted in a loss of crimp structure and uniformity of staple form, leading to a somewhat fuzzy, open staple. Earlier reports of fleece mosaicism (Ross 1933; Bosman 1935; Kelley 1939) also noted that the abnormal wool had longer and less discrete staples. These features are illustrated in Plate 1, Figure 1, which shows a

crossbred sheep with extensive mosaicism, and in Plate 2, which shows abnormal (left) and normal (right) staples from a number of fleece mosaics.

It is intended shortly to organize a search for "short-stapled" mosaics and this should indicate whether or not there is a real difference in the frequency of "long" and "short" staple fleece mosaicism.
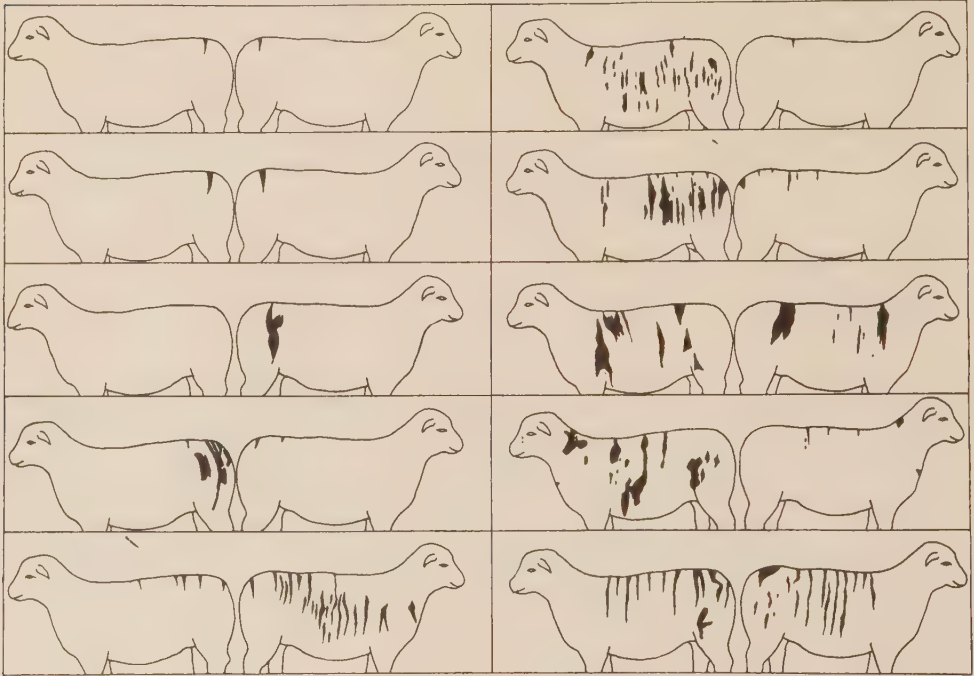


Fig. 1.—Patterns of fleece mosaicism.

### (b) Patterns of Mosaicism

The patterns of mosaicism were sketched after regrowth of the fleece allowed clear identification of the abnormal and normal areas. Drawings were made on a standard sheep silhouette. The position, number, and relative size of abnormal regions was checked carefully by two independent observers. Although the position and size of abnormal regions was subjectively assessed, several sheep were checked by direct tracing, and the error did not exceed 20 per cent. of the measured area of any single abnormal patch.

### III. Results and Discussion

The patterns of distribution of the areas of abnormal fleece type are illustrated in Figures 1 and 2 for 15 sheep from our collection. It can be seen that the abnormal wool nearly always occurs in more than one patch. This separation of the abnormal wool into a number of separate areas is a characteristic feature of fleece mosaicism. It might have been expected from text-book references to genetic mosaics that the abnormal area would be symmetrical and unitary, though several detailed descriptions of genetic mosaicism have previously demonstrated the inaccuracy of this idea. Many

coat-colour mosaics in mice, rats, rabbits, and guinea pigs have involved a single patch of abnormally coloured fur (Castle 1922, 1929; Pincus 1929; Fisher 1930; Bittner 1932) but several with more than one abnormally coloured area have been described (Wright and Eaton 1926; Dunn 1934; Feldman 1935).

Sturtevant (1929) concluded from his extensive studies of genetic mosaics in *Drosophila simulans* that the first cleavage patterns are indeterminate since there was no definite pattern among these mosaics. This indicated that cleavage nuclei are distributed to the blastoderm differently in different embryos. Detailed analyses showed that a complex pattern of migration was involved in the determination of the
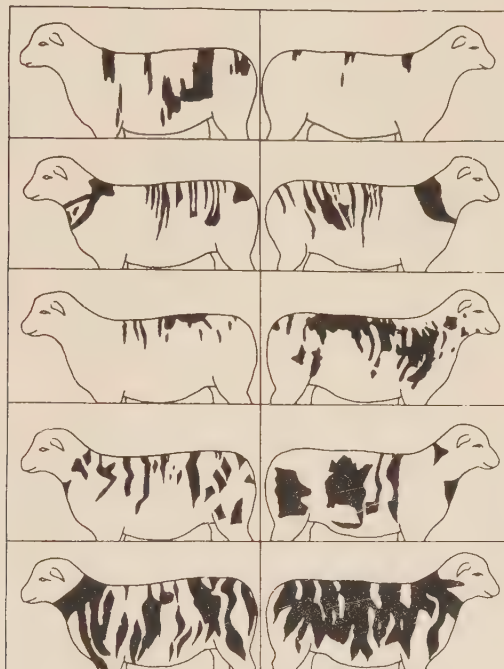


Fig. 2.—Patterns of fleece mosaicism.

imaginal discs, and of the relation of the imaginal discs to the surface of the adult. Patterson and Stone (1938) agreed with Sturtevant's conclusions. The main point of the evidence from *Drosophila* mosaics in so far as sheep mosaics are concerned is their clear demonstration that initially unitary mutational events may lead to widely separated mutant areas.

Although the differences between normal and abnormal wool in our sheep mosaics vary slightly from region to region, this variation is in accord with the normal gradients of expression of wool characters which occur over the body. These slight variations do not conflict with the close similarity of the basic differences in several regions; regardless of their anatomical position abnormal patches all differ from normal regions in the same ways and to essentially the same degree. This is illustrated in Plate 1, Figure 2, a Southdown mosaic in which the marked increase of length of

the wool on the abnormal regions has been shown to be associated with an equally marked decrease in the density of the follicle population. This similarity from region to region agrees with the hypothesis that the separate areas have a common origin in a single region of abnormal cells which later forms separate regions due to cell migrations.

These patterns point to the occurrence of two distinct processes during development: (1) an extension in the anteroposterior direction which is accompanied by intermigrations, along dorsoventral axes, of cells of the abnormal and normal regions. This process is deduced from the occurrence of the abnormal regions in separate patches aligned in the anteroposterior axis; (2) an extension, unaccompanied by any marked migration, which results in the separate regions being "stretched" in the dorsoventral direction. It is possible that this latter mechanism is responsible for the similar dorsoventral disposition of skin folds in some Merino sheep (Plate 1, Fig. 3).

The allometry of the sheep foetus has not been studied earlier than 45 days of gestation. Stephenson (personal communication) has shown that variation of allometric constants after this age is small. It may therefore be concluded that the processes of extension and migration deduced from the patterns of fleece mosaicism occur before 45 days of gestation. Regardless of the mechanism of the separation of an initial single region into a number of separated areas in the adult, it is clear that such separation does not constitute an argument against the hypothesis that fleece-type mosaicism is genetic in origin.

### (a) Extent of the Abnormal Areas

The total areas of the abnormal regions on each sheep, though subjectively assessed, indicate a mutational origin. These areas are given as percentages in Table 1.

The totals range from 0·2 to 44 per cent. and there is an indication that they form a binary sequence such as would occur if a mutational event happened at the first, second, third, etc. cell division. However, the sums of the areas of abnormal regions need not necessarily follow a binary sequence, for if the mutational event is *bidirectional*, therefore affecting both daughter cells, the cell lineages will comprise three genetic types: a normal and two mutant types. Non-disjunction of a chromosome at mitosis is a typical bidirectional mutation—it can result in one daughter cell being trisomic, the other monosomic. The cell lineage will then contain trisomic, disomic, and monosomic cells. Since mosaicism comprising three different variants of a tissue have not been observed either here or elsewhere it must be concluded that *either* the mutational basis of genetic mosaicism is unidirectional, so affecting only one of a pair of daughter cells, *or*, if it is bidirectional, then one of the mutant cells lineages is indistinguishable from normal or is inviable.

In the event of a third cell type being inviable, the total areas of mosaic regions will not follow a binary sequence. The percentages of abnormal tissue would follow the series 100, 33·3, 14·2, 6·7, 3·2 per cent., etc. as compared with the series 50, 25, 12·5, 6·3, 3·1 per cent., etc. It would be difficult to distinguish these series except in the second term, i.e. when the mutation occurred during the second cell division. In the bidirectional case this would result in 33·3 per cent. of abnormal tissue, whereas in the unidirectional case it would result in 25 per cent. of abnormal

tissue.  It is impossible to decide between these two alternatives on the data from our present group of mosaics, but the occurrence of one with 44 per cent. and three with 21–24 per cent. indicate fairly strongly that the causal basis is unidirectional.

The most probable types of unidirectional mosaicism are (i) gene mutation, and (ii) chromosome breakage.  The latter is initially bidirectional, but since the fragment lacking a centromere would soon be lost, it is effectively unidirectional.

### (b) Boundaries between Abnormal and Normal Regions

Regional variation of fleece structure seldom, if ever, exhibits sharp boundaries. The fleeces of some carpet-type sheep have an obvious division into an anterior region growing long wool and a posterior region growing carpet wool.  In a detailed examination of such an animal the authors have found that the boundary is quite diffuse, occupying at least several centimetres over which a gradation occurred from long wool to carpet wool.

TABLE 1

PERCENTAGE AREA OF ABNORMAL WOOL TYPE (STANDARD SILHOUETTE AREA = 100)

| Sheep No. | Left Side | Right Side | Mean | Sheep No. | Left Side | Right Side | Mean |
|---|---|---|---|---|---|---|---|
| 1 | 37·0 | 51·8 | 44·4 | 12 | 4·2 | 3·2 | 3·7 |
| 2 | 26·8 | 22·0 | 24·4 | 13 | 0·1 | 7·3 | 3·6 |
| 3 | 17·0 | 26·4 | 21·6 | 14 | 0·0 | 5·6 | 2·8 |
| 4 | 27·2 | 15·7 | 21·4 | 15 | 5·4 | 0·1 | 2·7 |
| 5 | 15·8 | 3·2 | 9·5 | 16 | 2·4 | 2·4 | 2·4 |
| 6 | 16·7 | 1·8 | 9·2 | 17 | 4·1 | 0·2 | 2·2 |
| 7 | 8·0 | 7·4 | 7·7 | 18 | 0·9 | 3·4 | 2·1 |
| 8 | 8·4 | 6·4 | 7·4 | 19 | 0·2 | 3·4 | 1·8 |
| 9 | 10·4 | 1·1 | 5·6 | 20 | 0·0 | 3·1 | 1·6 |
| 10 | 5·2 | 5·3 | 5·3 | 21 | 0·5 | 0·6 | 0·6 |
| 11 | 7·6 | 0·7 | 4·2 | 22 | 0·2 | 0·1 | 0·2 |

The boundary between abnormal and normal regions in mosaic fleeces is dramatically sharp—in the architecture of the staple, in the population of skin follicles and glands, and in the structure of the follicles.  These differences are illustrated in the photographs of wool and vertical sections of skin taken across the normal–abnormal boundary of a Merino mosaic (Plate 3, Figs. 1 and 2).

The sharpness of the boundaries between abnormal and normal regions supports the argument that these mosaics are genetic in origin even though in laboratory animals genes are known which cause "developmental" mosaicism of coat and skin colour with equally sharp boundaries (e.g. the spotting gene in mice).

### (c) Inheritance of Fleece Mosaicism

Genetic analyses in sheep can rarely be concluded in less than 4 years, and usually take much longer.  Therefore a rigorous test of the inheritance of fleece mosaicism is unlikely to be concluded until well after the death of the animals concerned.  However, some tests have been made.  Ten lambs have been produced

from matings between mosaic-fleeced parents, approximately 40 lambs have be produced from matings of mosaic rams and normal, unrelated ewes, and 13 lambs have been produced from mosaic ewes mated to normal rams. All of these lambs grew normal birthcoats and first-year fleeces. Such results would not disprove the inheritance of mosaicism as a pattern of the spotting type, but they argue strongly that it is unlikely.

A number of our mosaic sheep have come from small flocks of shortwools in which, if mosaicism were due to a single gene, other similar animals would be expected. In every case, the mosaic sheep was the only known case in the history of the flock.

The number of Merino sheep in our collection of fleece mosaics allows an estimate of the frequency of mosaicism in this breed. We have collected 12 mosaics in 2 years from a population of the order of 100 million giving an estimate of the frequency of mosaics in the order of $10^{-7}$. If mosaicism is determined by a single gene then the frequency of mosaics would be expected to be of the order of mutation frequencies *if* the gene is completely lethal. There is no indication from our sheep of any effect on viability either in themselves or in their progeny. This does not exclude a lethality imposed by selection against mosaics as parents. However, such selection is not practised universally since eight of the 11 rams in our collection were in use as stud or flock rams at the time of their identification. Three of these were Merinos, 1 Polwarth, and 4 shortwools (2 Southdown, 1 Dorset Horn, and 1 Ryeland). Selection against mosaicism in ewes would be much less stringent than in rams. It can be concluded that selection against mosaicism is not marked and it follows that if it is determined by a single gene then the expected frequency would be much higher than that actually found, i.e. the expected frequency on the hypothesis of a single gene would be greater than $10^{-5}$–$10^{-4}$ rather than the value of $10^{-7}$ actually found, which, even allowing our present figures to be a considerable underestimate of the frequency of mosaics, agrees with that expected on a hypothesis of somatic mutation.

## IV. CONCLUSIONS

It is not possible to demonstrate that fleece mosaics have a somatic genetic basis but there is strong circumstantial evidence to suggest that this is the correct interpretation, namely: (1) the probable binary sequence of the areas of abnormal regions; (2) the extremely sharp boundary between the normal and abnormal wool types and the underlying skin; (3) their frequency of occurrence being of the order of mutation frequencies; and (4) the indication that mosaicism is not inherited. As this project continues and more data are obtained, it is probable that accurate evaluation of (1) will allow more confidence to be placed in our interpretation.

Interest in sheep mosaic for fleece type lies primarily in their use as tools for the analysis of correlations between components of the fleece. Previously the presence or absence of such correlations has been determined from studies of groups of related sheep, i.e. of the same breed in a single flock. This is the "between sheep" level of comparison (Turner 1956). A weakness of this method is that correlations between characters may not be due to developmental interaction or to a common genetic basis, but rather to a common environmental effect.

A second approach has involved comparisons "between groups" of animals of different breeds, and here it is assumed that averaging over groups reduces or removes environmental effects which might affect correlations between characteristics of the fleece (see Daly and Carter 1956; Fraser, Short, and Carter, unpublished data).

Another approach involves the estimation of genetic correlations in a large group using individual pedigrees (Morley 1953; Schinckel, personal communication), or between groups which have been selected from a single original population in different directions for the traits in question (Morley 1953, 1955; Turner 1956). The latter method, utilizing selection lines, suffers from the disability that each pair of selection lines effectively allows only a single comparison, regardless of the numbers of sheep involved.

Studies of sheep mosaic for fleece type are useful since in comparisons between areas growing normal and abnormal wool, environmental differences can be regarded as trivial. Further, the genetic causation is identical in the separate abnormal regions and so, by comparing the differences between adjacent normal and abnormal areas in various regions of the body, it is possible to detect the effects of variation in the normal level of expression of a particular character, e.g. differences due to a gradient of fibre density over the body.

In later papers of this series comparisons of normal and abnormal wool from a number of mosaic sheep will be detailed and discussed on the assumptions (*a*) that fleece mosaicism is "genetic" in causation, and (*b*) that the mutation is probably a deletion.

## V. Acknowledgments

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STUDIES OF SHEEP MOSAIC FOR FLEECE TYPE. I



Fig. 1.—Crossbred sheep showing extensive (44 per cent.) fleece mosaicism.

Fig. 2.—Southdown fleece mosaic showing similarity of abnormal wool in different regions.

Fig. 3.—Merino sheep showing dorsoventral disposition of skin folds.

STUDIES OF SHEEP MOSAIC FOR FLEECE TYPE. I
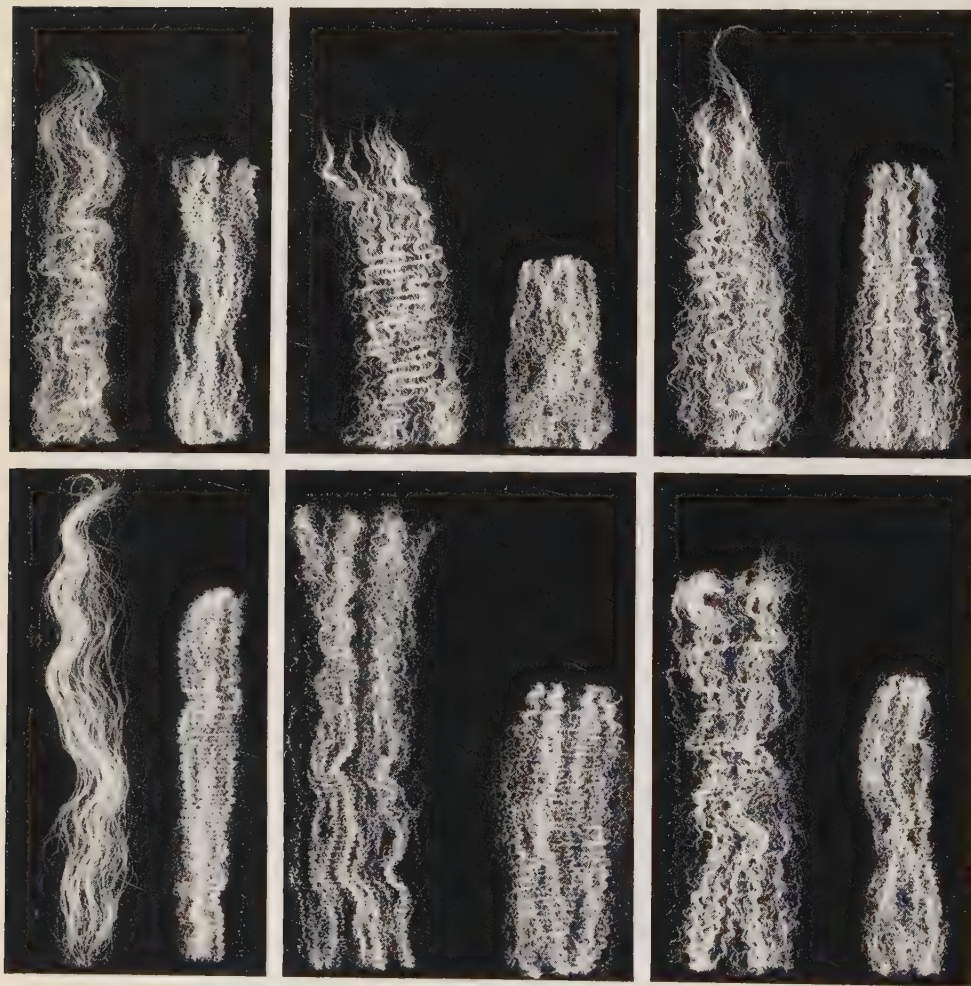


Wool staples from mosaic-fleeced Merino sheep.  Abnormal staples on the left, normal staples
on the right of each pair.

STUDIES OF SHEEP MOSAIC FOR FLEECE TYPE. I
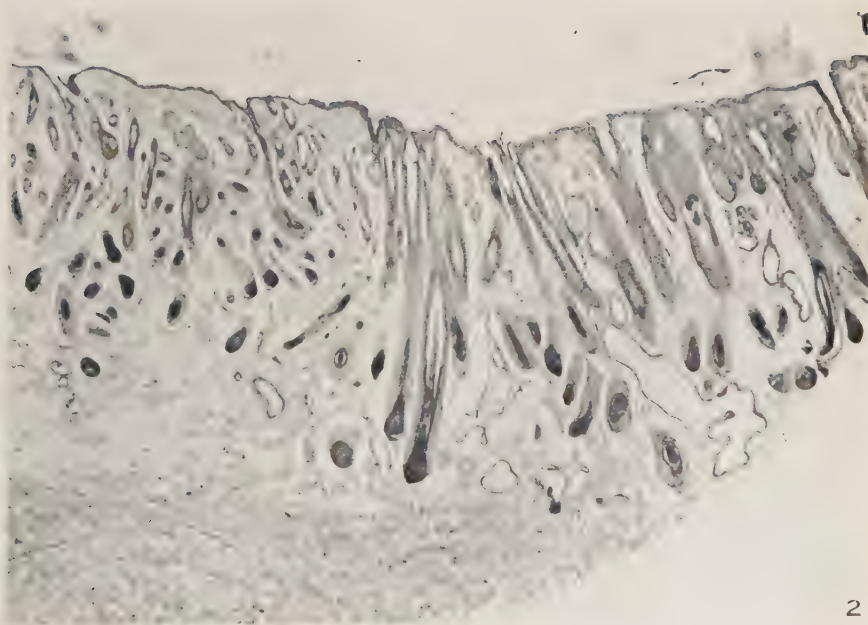


Fig. 1.—Wool staple differences in a Merino fleece mosaic.

Fig. 2.—Photomicrograph of a vertical section across the boundary between normal and abnormal regions of skin. Note difference in follicle depth and size of suderiferous glands.

# FUNGAL CELLULASES

## IX. Growth of Stachybotrys Atra on Cellulose and Production of a β-Glucosidase Hydrolysing Cellobiose

### By G. Youatt*

*Summary*

The growth of *Stachybotrys atra* on an improved Waksman-Carey medium containing cellulose as the sole carbon source is described. Under these conditions the organism produces an extracellular cellulase and an intracellular β-glucosidase which is capable of hydrolysing cellobiose. Some properties of this β-glucosidase are discussed.

## I. Introduction

Previous papers in this series have been concerned with the growth and production of enzymes by *Strachybotrys atra*. Thomas (1956) has described the production of an extracellular cellulase which is produced by the organism when grown on cellulose and which hydrolyses poly-β-1,4-glucose chains to a mixture of glucose and cellobiose but which is incapable of hydrolysing cellobiose itself. Jermyn (1955) has described an extracellular β-glucosidase which is produced when the organism is grown on glucose or starch, but which does not hydrolyse cellobiose. The fate of the cellobiose produced by the cellulase when the organism was grown on cellulose was therefore unknown. The present paper is concerned with some improvements in the culture of *S. atra* on media containing cellulose as the sole source of carbon and also with some properties of an intracellular β-glucosidase which is capable of hydrolysing cellobiose and cellulose oligosaccharides and which differs from the two enzymes already described.

## II. Methods

### (a) Determination of Fungal Growth

Since the presence of undigested cellulose prevented the determination of mycelial dry weights, the extent of fungal growth was estimated by the amount of insoluble nitrogen formed by the culture. The culture (50 ml) was filtered, and after washing the mycelium with *c.* 200 ml water, its nitrogen content was determined by the method of McKenzie and Wallace (1954). Growth of the organism is expressed as mg nitrogen formed per culture. If it is assumed that the mycelium contains 4·6 per cent. nitrogen, as found by Thomas (1956) for mycelium grown on glucose, then 1 mg of nitrogen is equivalent to 21·7 mg dry weight of mycelium.

### (b) Estimation of Cellulase Activity

The viscometric method of Thomas (1956), using sodium carboxymethylcellulose as substrate, was employed. It was found that over the restricted range of cellulase

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activities encountered in untreated culture filtrates the equation used by Thomas to relate enzyme concentration to rate of decrease of viscosity could be simplified and the following equation was used for calculating cellulase activities:

$$E = (\eta_{t=0})^{1.25}\,\mathrm{d}(1/\eta)/\mathrm{d}t,$$

where $E$ = enzyme concentration in units/g solution, and
$\quad$ $\eta$ = specific viscosity.

### (c) Estimation of Cellobiase Activity

The determination of cellobiase activity by measurement of the increase in reducing sugar titre is insensitive and a manometric method based on that of Keilin and Hartree (1948) was used. In this technique, the glucose formed by the hydrolysis of cellobiose was oxidized in the presence of glucose oxidase and the oxygen consumption measured by standard Warburg techniques. The Warburg flasks contained 1 ml McIlvaine sodium phosphate–citric acid buffer, pH 5·4, 1 ml. 1 per cent. w/v glucose oxidase (Sigma Chemical Co., U.S.A.), 0·5 ml 1 per cent. w/v catalase (L. Light & Co. Ltd., England), 0·1 ml ethanol, and 1 ml of the enzyme preparation; the side-arm of the flask contained 1 ml 4 per cent. w/v cellobiose solution. Measurements of the oxygen consumption, which under these conditions is directly proportional to time, were made at 5-min intervals. The rate of oxygen consumption was calculated by the method of Aldridge, Berry, and Davies (1949) and converted to mg glucose formed per minute. One unit of enzyme activity is that quantity of enzyme which liberates 1 mg of glucose per minute under these conditions.

### (d) Preparation of Enzyme Solutions

Cellobiase activity is found in the mycelium of *S. atra*; occasionally activity has been found in the medium but such occurrences have been sporadic and generally associated with old cultures—the enzyme is characteristically intracellular. Enzyme solutions were prepared by filtering off the mycelium, washing with water, and then grinding it with 10–20 ml of water in a Potter-Elvehjem homogenizer. The extract was filtered to remove debris and the clear solution used.

Culture filtrates were used as a source of cellulase without further treatment.

### III. Growth Experiments

### (a) Effect of Cultural Conditions

The fungus was maintained by serial transfer on cellulose–agar slopes (McQuade, unpublished data). Spore suspensions prepared from these slopes were used to inoculate 50-ml amounts of medium contained in 250-ml conical flasks. Incubation was at 28°C and the reciprocating shaker used had a total excursion of 2·5 in. and a rate of 98 c/min unless otherwise stated.

Work in this Laboratory has shown that greatly improved growth of *S. atra* can be obtained by modifying the original Waksman-Carey medium (McQuade, unpublished data). Though this work has been done using glucose as the carbon source it appeared reasonable to assume that similar improvements could also be

made with cellulose media. Accordingly an investigation was made of the effect of changes in the levels of cellulose, nitrogen, phosphate, and shaking rate on the growth and enzyme production of the fungus. The minor constituents of the medium, which appeared to be at adequate concentrations, were not investigated.

TABLE 1

EFFECT OF CELLULOSE CONCENTRATION AND SHAKING RATE ON THE GROWTH
OF S. ATRA AFTER 7 DAYS

| Cellulose (g/l) | Mean Mycelial Nitrogen (mg) | Shaking Rate (c/min) | Mean Mycelial Nitrogen (mg) |
|---|---|---|---|
| 5 | 3·87 | 98 | 7·70 |
| 10 | 5·31 | 120 | 4·36 |
| 20 | 8·64 | | |

In order to examine the effect of changes in the concentration of media constituents, a number of flasks representing the various treatment combinations were set up in factorial arrangements (Cochran and Cox 1950), and determinations made of the extent of growth and level of enzyme production. Table 1 shows the increasing amount of growth with increasing cellulose concentration and the deleterious effect of a high rate of shaking.

TABLE 2

EFFECT OF CELLULOSE CONCENTRATION AND AGE OF CULTURE ON THE GROWTH
AND ENZYME PRODUCTION OF S. ATRA

| | Mean Mycelial Nitrogen (mg) | Mean Cellulase Activity | Mean Cellobiase Activity |
|---|---|---|---|
| Cellulose (g/l) | | | |
| 10 | 6·77 | 18·40 | 0·26 |
| 20 | 11·72 | 24·18 | 0·39 |
| Age (days) | | | |
| 6 | 8·04 | 10·37 | 0·41 |
| 12 | 9·36 | 19·13 | 0·24 |
| 18 | 10·33 | 31·84 | |

Effects due to ammonium chloride and dipotassium hydrogen phosphate were observed in those experiments in which the levels of these salts were in the range 5–10 g/l and 3–6 g/l respectively. However, when the concentration of ammonium chloride was decreased (2·5–5 g/l) and that of potassium phosphate increased (6–12 g/l) no effects attributable to these salts were observed.

In order to determine whether the effects of changes in the composition of the medium were influenced by the age of the culture, an experiment was carried out in which the cultures were harvested after 6, 12, and 18 days. The levels of ammonium

chloride and potassium phosphate were in the ranges previously found to be optimal, and under these conditions it was found that the extent of growth was dependent only on the concentration of cellulose and the length of time for which the culture was allowed to grow (Table 2).

The effects of these various treatments on the yield of enzymes were very similar to those produced in growth. Production of cellulase appeared to run parallel with growth and, at the concentration of ammonium chloride and potassium phosphate finally chosen, the factors influencing the yield were cellulose concentration and age of the culture. Similar results were found with cellobiase except that, in contrast with cellulase, the yield of enzyme decreased with age of the culture.
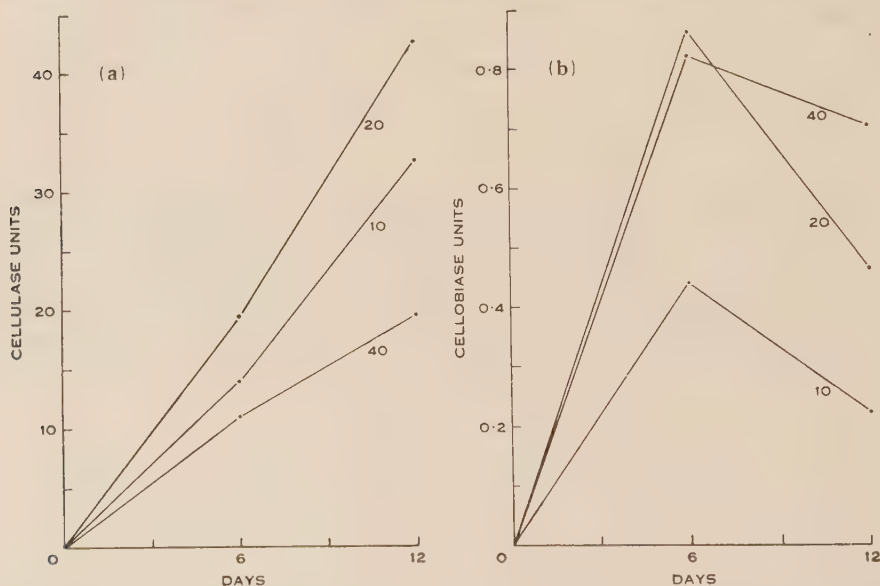


Fig. 1.—Production of (a) cellulase and (b) cellobiase by *S. atra* in media containing high levels of cellulose. Enzyme activities refer to the total activity of the culture and the cellulose concentrations (g/l) are given on the figures.

Thus the following standardized medium was chosen as being the most suitable for the general growth of *S. atra* on cellulose and for the production of the cellulolytic enzymes:

|             | g/l  |                    | mg/l  |
|-------------|------|--------------------|-------|
| Cellulose   | 20   | $CaCl_2$           | 20    |
| $NH_4Cl$    | 5    | $ZnSO_4.7H_2O$     | 2     |
| $K_2HPO_4$  | 7·5  | $MnSO_4.4H_2O$     | 1     |
| $MgSO_4.7H_2O$ | 1 | Iron-alum          | 10    |
|             |      | Biotin             | 0·02  |

### (b) *Enzyme Production in the Presence of High Concentrations of Cellulose*

Thomas (1956) reported a diminished yield of cellulase when the amount of cellulose in the medium was greater than 2 g/l. Since no evidence of this was obtained

during the experiments just described, an experiment was carried out to see if this effect could be observed at higher levels of cellulose. The results are shown in Figures 1(a) and 1(b). The concentration of cellulose in the medium may be raised to over 20 g/l before there is any decrease in the amount of cellulase detectable. Cellobiase production appears to be relatively unaffected by this high level of cellulose.

### (c) Enzyme Production during the First 12 Days of Growth

Growth of the organism on this medium is fairly rapid and the cultures mature at about 7 days. Figure 2 illustrates the growth, production of enzymes, and pH of the medium during a 12-day growth period.
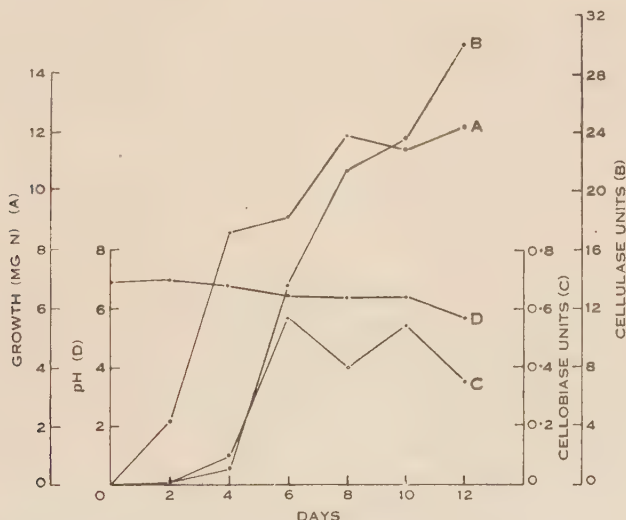


Fig. 2.—Growth and enzyme production of *S. atra* on cellulose. *A*, growth (mg N); *B*, cellulase activity of the medium; *C*, cellobiase activity of mycelium; *D*, pH of the medium.

### IV. Properties of Cellobiase

### (a) Adaptive Nature of Cellobiase

Since cellulase activity is not found when *S. atra* is grown on glucose and the β-glucosidase of Jermyn is not found in cultures growing on cellulose it was of interest to examine the production of cellobiase under these two sets of conditions. It was found that it is only when cellobiose, either added as such or produced by the breakdown of cellulose, is present that the enzyme is produced in any great quantity (Table 3).

### (b) Extractability of the Enzyme

Though grinding of the mycelium with water in a Potter-Elvehjem homogenizer was used as the standard technique for extracting the enzyme, other methods were tried. Of these, grinding the mycelium with silica flour and water in a mortar gave equally active extracts but dispersing the mycelium in a Waring Blendor, with or without the addition of abrasives, gave lower yields. It was also possible to freeze-dry the mycelium and extract the resulting powder.

An attempt was made to effect a partial purification of the enzyme by selective extraction of the mycelium with cold dilute ethanol. Freeze-dried mycelium (200 mg) was ground at 5°C with 10-ml aliquots containing 1 ml of 0·1M acetate buffer, pH 5, and ethanol to give concentrations of 0, 10, 20, and 40 percent. v/v.

TABLE 3

PRODUCTION OF CELLOBIASE BY S. ATRA ON VARIOUS CARBON SOURCES

| Carbon Source | Cellobiase Activity of Culture | Mycelial Weight (mg) |
|---------------|-------------------------------|----------------------|
| Cellulose     | 0·59                          | 270*                 |
| Glucose       | 0·04                          | 273                  |
| Maltose       | 0·02                          | 265                  |
| Starch        | 0·01                          | 239                  |
| Cellobiose    | 0·66                          | 320                  |

*Calculated value assuming 4·6 per cent. nitrogen in mycelium.

The mycelial debris was removed by centrifugation and the supernatants assayed for cellobiase activity. Table 4 lists the activities of the extracts and also the amounts of protein extracted, using an arbitrary scale deduced from the optical densities of the solutions at 275 m$\mu$. From these results it appears that by extraction of the mycelium with 20 per cent. ethanol, discarding the extract, and re-extracting the mycelium with 10 per cent. ethanol it should be possible to obtain c. 85 per cent. of the enzyme activity with only c. 25 per cent. of the total extractable protein, a purification factor of about 4. It was found, however, that when the mycelium from 2 l. of culture fluid was fractionated between 10 and 20 per cent. ethanol in this way, it gave rise to only 5 mg of crude protein. In view of this very small amount of material further fractionation experiments were not attempted.

TABLE 4

FRACTIONAL EXTRACTION OF CELLOBIASE FROM THE MYCELIUM OF S. ATRA

| Percentage ethanol | 0 | 10 | 20 | 40 |
|--------------------|------|------|------|------|
| Cellobiase activity of extract | 0·64 | 0·58 | 0·05 | 0·02 |
| Protein in extract (arbitrary units) | 1·00 | 0·68 | 0·41 | 0·62 |

(c) *Hydrolysis of Various Glycosides*

Mycelial extracts prepared in this way hydrolysed cellobiose and $p$-nitrophenyl $\beta$-glucoside readily but had only slight activity towards methyl $\beta$-glucoside. There was only a trace of $\alpha$-glucosidase activity as shown by action against $p$-nitrophenyl $\alpha$-glucoside but in spite of this there was considerable hydrolysis of $p$-nitrophenyl $\alpha$-cellobioside. However, the hydrolysis of the cellobioside does not go to completion and if a correction is made for the slight $\alpha$-glucosidase activity it appears that only

one mole of glucose is being liberated per mole of cellobioside. The $\beta$-linkage between the glucose residues is readily split but the $\alpha$-linkage of the aglucone is almost completely resistant. Lactose, which differs from cellobiose solely in the configuration of the terminal $C_4$ hydroxyl, is essentially unattacked.

### (d) Effect of Time and Enzyme Concentration

Under the conditions given above for the assay of the enzyme the rate of hydrolysis of cellobiose is linear with time and proportional to enzyme concentration (Figs. 3(a), 3(b)).
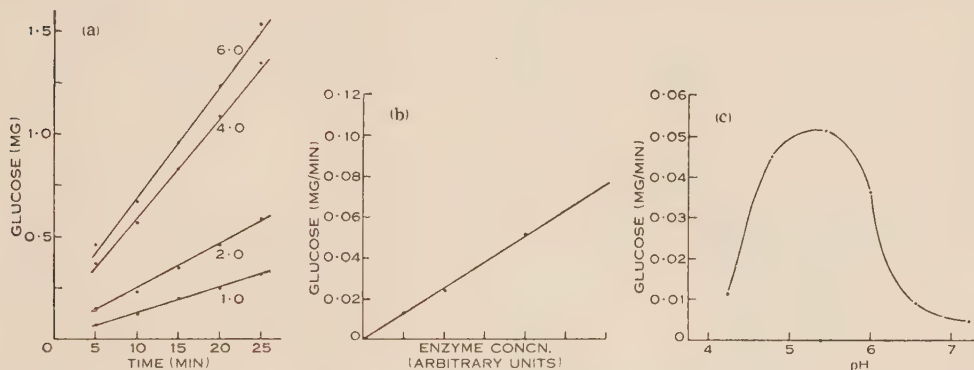


Fig. 3.—(a) Hydrolysis of cellobiose by various concentrations of cellobiase; (b) effect of enzyme concentration on the rate of hydrolysis of cellobiose by cellobiase; (c) pH–activity curve for cellobiase.

### (e) Effect of pH

In Figure 3(c) is set out the pH–activity curve for the hydrolysis of cellobiose. McIlvaine buffers were used throughout this experiment as they covered the required pH range. The limits of this pH curve may be affected by the pH–activity relationships of the glucose oxidase used in the assay system. Since, however, the rate of hydrolysis at the optimum pH observed is within the range where the cellobiase concentration is the limiting factor, this optimum will not be influenced by the other enzymes in the system.

### (f) Effect of Substrate Concentration

The relation between the substrate concentration and the rate of hydrolysis for the two substrates cellobiose and $p$-nitrophenyl $\beta$-glucoside were investigated. The results lead to rectilinear plots according to the method of Lineweaver and Burk and the Michaelis constants were found to be $3.9 \times 10^{-4}$M and $2.0 \times 10^{-4}$M respectively. The extracellular $\beta$-glucosidase has a Michaelis constant of $3 \times 10^{-5}$M when acting on $p$-nitrophenyl $\beta$-glucoside under comparable conditions (Jermyn 1955).

### (g) Hydrolysis of Cellulose Oligosaccharides

It was found that the oligosaccharides from cellobiose to cellopentaose inclusive and also a cellodextrin of mean chain length 11, which was prepared by sulphuric acid

degradation of cellulose, were readily hydrolysed and at comparable rates.  Some of the results are given in Figure 4.  Attempts to demonstrate the hydrolysis of cellulose itself, either in the form of alkali-swollen cellulose or as a soluble derivative such as carboxymethylcellulose were inconclusive.
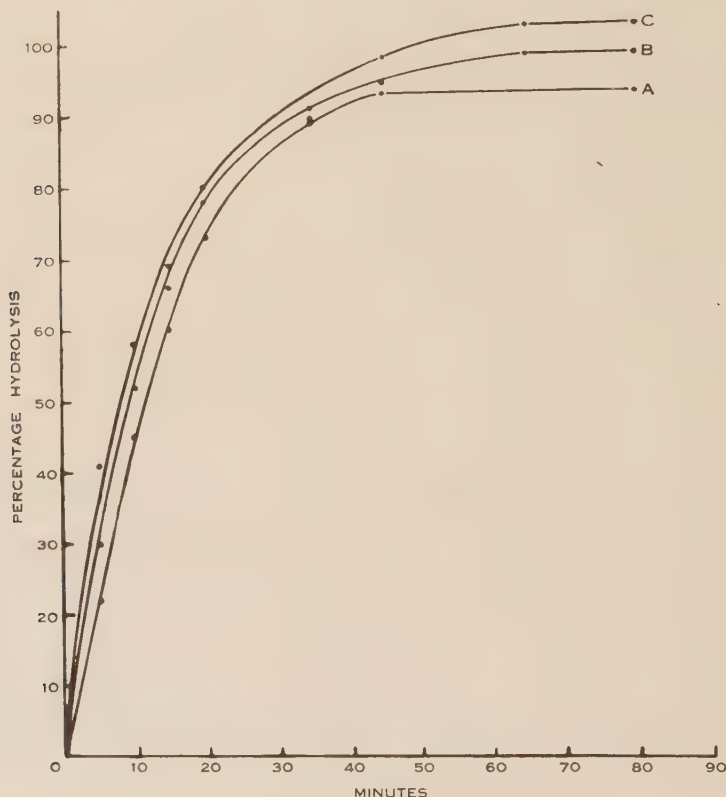
Fig. 4.—Hydrolysis of cellobiose (A), cellotetraose (B), and a cello-
dextrin of chain length 11 units (C) by S. atra cellobiase.

## V. Discussion

Compared with the original Waksman-Carey medium used there has been about a sixfold increase in yield of mycelium.

Correlation of the results of this paper with previous ones in the series leads to the recognition of at least three well-defined enzymes in S. atra which are capable of hydrolysing the $\beta$-1, 4-glucosidic linkage, but which differ in their specificity towards the rest of the substrate molecule.  They are (1) $\beta$-glucosidase (Jermyn 1955) which attacks aryl $\beta$-glucosides but not cellobiose or cellobiosides; (2) cellulase (Thomas 1956) which attacks chains of $\beta$-1, 4-linked glucosaccharides having a chain length of at least three units, and soluble cellulose derivatives; (3) cellobiase which hydrolyses cellobiose, $\beta$-1,4-linked glucosaccharides up to a chain length of at least 11 units, and some aryl $\beta$-glucosides.

The production of cellulase is very closely linked with the production of mycelium, and the finding that proportionately more cellulase is produced as the age of the culture increases may not be due so much to increased production of the enzyme as to the liberation of absorbed enzyme from the diminishing amount of cellulose in the medium.

Since the cellobiase is capable of hydrolysing a cellodextrin and on this basis could be classified as a cellulase, it is difficult to account for the failure to demonstrate any hydrolysis of either swollen cellulose or carboxymethylcellulose and it is not possible to set any upper limit to the number of units of a $\beta$-1, 4-linked glucosaccharide chain beyond which it is not attacked by the enzyme. However, under the normal physiological conditions of growth when the enzyme is within the mycelium only those oligosaccharides sufficiently small to pass through the cell wall would be presented to it and it seems unlikely that a molecule of any great chain length would be involved.

## VI. Acknowledgments

I am extremely grateful to Mr. W. B. Hall for the statistical work required in this paper, to Mr. A. B. McQuade for allowing me to draw upon his extensive knowledge of the growth habits of *S. atra*, and to Dr. M. A. Jermyn for the cello-oligosaccharides.

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# APPLICATION OF SAMPLING VARIABLES IN THE IDENTIFICATION OF METHODS WHICH YIELD UNBIASED ESTIMATES OF GENOTYPIC VARIANCE COMPONENTS

By B. Griffing*

*Summary*

This study attempts to develop a logical basis for determining those experimental methods which give unbiased estimates of population variances. The steps involved are: (1) definition of the population and parameters; (2) definition of sampling and experimental procedure; (3) construction of the mathematical model; (4) derivation of expectations of mean squares; and (5) demonstration that unbiased estimates can be obtained from the observed mean squares.

This study considers problems involving sampling with replacement from finite populations whose elements are not of equal frequency. Use is made of "sampling" or "dummy" variables in the construction of the mathematical models.

Three examples are described, and in each example the objective is to estimate general and specific combining ability variances of random mating populations. In each example sampling with replacement is carried out among members of inbred populations. These conceptual inbred populations are derived from their parental random mating populations by inbreeding without selection. The experimental material consists of crosses among the sample inbreds.

In the first example, two independent samples are drawn with replacement from the inbred population $\pi$ which is derived from the random mating population II. The experimental material consists of hybrids between the inbreds of one sample and those of the other. It is shown that unbiased estimates of the general and specific combining ability variances of the random mating population II are obtainable from the observed mean squares.

In the second example, or series of examples, only one sample is drawn from $\pi$. Crosses among the sample inbreds constitute the experimental material. Four variants of this general diallel procedure are investigated. It is shown that in those diallel crossing systems in which the parental lines are included in the analysis, unbiased estimates of the variances cannot be obtained. However, in the so-called "modified" diallel systems, in which the parental lines are not included, unbiased estimates can be obtained.

In the last example, one sample is drawn with replacement from each of two inbred populations $\pi_1$ and $\pi_2$. These inbred populations are derived from two different random mating populations $II_1$ and $II_2$ by inbreeding without selection. The experimental material consists of the hybrids between the inbreds of the two samples. It is demonstrated that unbiased variance estimates can be obtained for the population $II_3 = \pi_1 \times \pi_2$ but not for variances of $II_1$ or $II_2$.

## I. Introduction

The purpose of this study is to provide a logical basis for using certain experimental procedures to obtain unbiased estimates of population parameters. More explicitly, we are concerned with the estimation of genotypic variance components

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of random mating populations. In some cases it seems intuitively quite clear what experimental and analytical procedures are appropriate. In other cases the choice is much more difficult.

We wish, then, to set out and illustrate the steps which are necessary in order to provide a logical basis for determining those methods which give unbiased estimates of population variances. The steps are:

(i) Define the population and parameters about which inferences are to be made.

(ii) Define the sampling procedure and experimental method.

(iii) Construct the mathematical model.

(iv) Derive the expectations of mean squares for the appropriate analysis of variance.

(v) Show that unbiased estimates of population variances can be made from the observed mean squares.

The argument may be greatly simplified by use of the so-called "sampling" or "dummy" variables due to Cornfield (1944). Kempthorne (1952, 1957) and Wilk and Kempthorne (1955, 1956) have used these variables elegantly in clarifying and generalizing many analysis of variance problems in which sampling is made without replacement from finite populations. The elements in a given population are generally assumed to have equal frequencies. In the present case we shall use the sampling variables for genetic problems in which random sampling is made with replacement from finite populations. The elements in a given population are not assumed to have equal frequencies.

The general procedure outlined here can be used for a great variety of genetic problems. However, we shall confine our attention to three rather closely related examples which involve the estimation of general and specific combining ability variances from random mating populations. All three examples utilize inbred lines in a somewhat involved experimental approach. We assume that a population of homozygous lines can be derived from a given random mating population. Sampling is then carried out among the elements of this conceptual population. Crosses among the sample inbreds yield hybrid material which provides the observational basis from which inferences are made about the original random mating population.

In the first two examples a single random mating population is involved. In the last example we consider a situation involving two random mating populations.

## II. Examples Involving a Single Random Mating Population

### (a) Definition of Population Parameters

Consider a random mating population in equilibrium, the zygotic array of which we can designate as $\Pi$. By imposing an inbreeding system without selection on the elements of $\Pi$ we obtain an inbred population the array of which we denote as $\pi$. Note that $\Pi$ can be generated by "squaring" $\pi$. We characterize the different

genotypes in the inbred population by the finite set $S = I_1, I_2, \ldots, I_p$, which has the associated set of frequencies $F = f_1, f_2, \ldots, f_p$ such that $\sum_i f_i = 1$.

In this section we consider two examples which involve different sampling procedures. First, we examine the sampling method by means of which two independent random samples are drawn with replacements from $S = I_1, \ldots, I_p$. We arbitrarily designate the "$a$" inbreds of one sample as $L_1, L_2, \ldots, L_a$, and the "$b$" inbreds of the other sample as $T_1, T_2, \ldots, T_b$, where $a$ and $b$ need not be the same numbers. All possible crosses are made between the $L$'s and the $T$'s to provide the experimental material on which the observations are made.

TABLE 1

A REPRESENTATION OF THE RANDOM MATING POPULATION, $\Pi$

|  | $I_1$ | $I_2$ | $I_3$ | $\ldots$ | $I_P$ |  |
|---|---|---|---|---|---|---|
|  | $(f_1)$ | $(f_2)$ | $(f_3)$ | $\ldots$ | $(f_P)$ |  |
| $I_1$ | $I_1I_1$ | $I_1I_2$ | $I_1I_3$ | $\ldots$ | $I_1I_P$ |  |
| $(f_1)$ | $\tau_{11}$ | $\tau_{12}$ | $\tau_{13}$ | $\ldots$ | $\tau_{1P}$ | $\tau_{1.}$ |
| $I_2$ | $I_2I_1$ | $I_2I_2$ | $I_2I_3$ | $\ldots$ | $I_2I_P$ |  |
| $(f_2)$ | $\tau_{21}$ | $\tau_{22}$ | $\tau_{23}$ | $\ldots$ | $\tau_{2P}$ | $\tau_{2.}$ |
| $\vdots$ |  |  |  |  |  | $\vdots$ |
| $I_P$ | $I_PI_1$ | $I_PI_2$ | $I_PI_3$ | $\ldots$ | $I_PI_P$ |  |
| $(f_P)$ | $\tau_{P1}$ | $\tau_{P2}$ | $\tau_{P3}$ | $\ldots$ | $\tau_{PP}$ | $\tau_{P.}$ |
|  | $\tau_{.1}$ | $\tau_{.2}$ | $\tau_{.3}$ | $\ldots$ | $\tau_{.P}$ | $\tau_{..}$ |

Second, we wish to consider a sampling method in which we draw at random with replacement only one sample of "$p$" lines from $S = I_1, I_2, \ldots, I_p$. The sample lines are arbitrarily designated as $I_1^*, I_2^*, \ldots, I_p^*$. Crosses are made among the $I^*$'s. Variations of this diallel method are due to the inclusion of different kinds of crosses in the experimental material. We consider four such variations.

Before considering the two examples it is necessary to define the parameters for which we wish the samples to yield unbiased estimates. The random mating population, $\Pi$, can be generated by making all possible crosses among members of the inbred population. This follows from the facts that the zygotic array can be obtained as the square of the gametic array, and that the gametic array and the array of inbreds are isomorphic. Thus $\Pi$ may be represented by the elements $I_mI_n$ in the body of Table 1. The frequency of $I_mI_n$ is $f_mf_n$, i.e. the product of the appropriate marginal frequencies. We denote the genotypic value of $I_mI_n$ as $\tau_{mn}$.

The population weighted row, column, and overall means are defined as follows:

$$\tau_{m.} = \sum_n f_n \, \tau_{mn} \, ,$$

$$\tau_{.n} = \sum_m f_m \, \tau_{mn} \, ,$$

$$\tau_{..} = \sum_m \sum_n f_m f_n \, \tau_{mn} \, .$$

The following identity in means exists:

$$\tau_{mn} \equiv \tau_{..} + (\tau_{m.} - \tau_{..}) + (\tau_{.n} - \tau_{..}) + (\tau_{mn} - \tau_{m.} - \tau_{.n} + \tau_{..}),$$

which may be written more conveniently as

$$\tau_{mn} = \mu + G_m + G_n + S_{mn} \, ,$$

where

$\mu = \tau_{..}$ is the population overall mean,

$G_m = (\tau_{m.} - \tau_{..})$ is the general combining ability (g.c.a.) effect for the $m$th inbred,

$G_n = (\tau_{.n} - \tau_{..})$ is the g.c.a. effect for the $n$th inbred,

and

$S_{mn} = (\tau_{mn} - \tau_{m.} - \tau_{.n} + \tau_{..})$ is the specific combining ability (s.c.a.) effect associated with the cross of the $m$th and $n$th inbreds.

The following restrictions hold for these effects:

$$\sum_m f_m \, G_m = \sum_n f_n \, G_n = 0,$$

$$\sum_m f_m \, S_{mn} = 0 \text{ for all } n,$$

and

$$\sum_n f_n \, S_{mn} = 0 \text{ for all } m.$$

The following identity in sums of squares exists:

$$\sum_m \sum_n f_m f_n \, \tau_{mn}^2 - \tau_{..}^2 \equiv 2 \sum_m f_m \, G_m^2 + \sum_m \sum_n f_m f_n \, S_{mn}^2 \, .$$

This partitioning can be symbolized as follows:

$$\sigma_G^2 = 2\sigma_{\text{g.c.a.}}^2 + \sigma_{\text{s.c.a.}}^2 \, ,$$

where

$\sigma_G^2 = (\sum_m \sum_n f_m f_n \, \tau_{mn}^2 - \tau_{..}^2)$ is the total genotypic variance,

$\sigma_{\text{g.c.a.}}^2 = (\sum_m f_m \, G_m^2)$ is the g.c.a. variance,

and

$\sigma_{\text{s.c.a.}}^2 = (\sum_m \sum_n f_m f_n \, S_{mn}^2)$ is the s.c.a. variance.

The objective of the experimental research is to estimate the two population variance components, $\sigma_{\text{g.c.a.}}^2$ and $\sigma_{\text{s.c.a.}}^2$ .

(b) *Example 1.—A Sampling Procedure Involving Two Independent Random Samples*

Two independent random samples are drawn with replacement from $\pi$. The elements in one sample are denoted as $L_1, L_2, \ldots, L_a$, and the elements in the other sample as $T_1, T_2, \ldots, T_b$. All possible crosses are made between the $L$'s and $T$'s and it is this set of hybrids that provides the experimental material.

For simplicity we consider only the completely randomized design in which there are "$c$" observations for each hybrid combination. Thus $x_{ijk}$ represents the $k$th observation of the hybrid resulting from the cross $L_i \times T_j$.

The notation used is as follows:
For sums:

$$X_{ij.} = \sum_{k} x_{ijk} ,$$

$$X_{i..} = \sum_{j}\sum_{k} x_{ijk} ,$$

$$X_{.j.} = \sum_{i}\sum_{k} x_{ijk} ,$$

and

$$X_{...} = \sum_{i}\sum_{j}\sum_{k} x_{ijk} .$$

For means:

$$x_{ij.} = \frac{1}{c} X_{ij.} ,$$

$$x_{i..} = \frac{1}{bc} X_{i..} ,$$

$$x_{.j.} = \frac{1}{ac} X_{.j.} ,$$

and

$$x_{...} = \frac{1}{abc} X_{...} .$$

The appropriate analysis of variance for this two-way classification is given in Table 2. We wish now to construct the mathematical model, and then to use the model to determine the expectations of mean squares in the analysis of variance. The objective is to show that these mean squares may be used to give unbiased estimates of the population variances.

(i) *Construction of the Model.*—In this section our primary concern is the treatment of the genotypic effects. Therefore, to simplify the presentation and to focus attention on this objective we shall neglect the error terms in construction of the model. We denote the genotypic value of the cross $L_i \times T_j$ as $x_{ij}$. The objective then is to characterize this variate in terms of the population genotypic effects.

Sampling variables are defined as follows:

$$\begin{cases} \delta_i^m = 1 \text{ if } I_m = L_i \\ \quad = 0 \text{ otherwise} \end{cases},$$

and

$$\begin{cases} \rho_j^n = 1 \text{ if } I_n = T_j \\ \quad = 0 \text{ otherwise} \end{cases}.$$

TABLE 2

ANALYSIS OF VARIANCE FOR EXAMPLE 1

| Source | D.F. | Sums of Squares* | Mean Squares |
|--------|------|------------------|--------------|
| Between $L$'s | $a-1$ | $S_l$ | $M_l$ |
| Between $T$'s | $b-1$ | $S_t$ | $M_t$ |
| $L \times T$ | $(a-1)(b-1)$ | $S_{lt}$ | $M_{lt}$ |
| Error | $ab(c-1)$ | $S_e$ | $M_e$ |

\* Where

$$S_l = \sum_i \frac{X_{i..}^2}{bc} - \frac{X_{...}^2}{abc}, \qquad S_t = \sum_j \frac{X_{.j.}^2}{ac} - \frac{X_{...}^2}{abc},$$

$$S_{lt} = \sum_i \sum_j \frac{X_{ij.}^2}{c} - \sum_i \frac{X_{i..}^2}{bc} - \sum_j \frac{X_{.j.}^2}{ac} + \frac{X_{...}^2}{abc}.$$

The expected mean values of these variables are

$$E(\delta_i^m) = f_m,$$

and

$$E(\rho_j^n) = f_n.$$

The expected values for the square and cross products of the $\delta$ and $\rho$ terms are as follows:

$$E(\delta_i^m \delta_i^m) = E(\delta_i^m) = f_m, \qquad E(\delta_i^m \delta_i^{m'}) = 0,$$

$$E(\delta_i^m \delta_{i'}^m) = f_m^2, \quad \text{and} \quad E(\delta_i^m \delta_{i'}^{m'}) = f_m f_{m'}.$$

$$E(\rho_j^n \rho_j^n) = E(\rho_j^n) = f_n, \qquad E(\rho_j^n \rho_j^{n'}) = 0,$$

$$E(\rho_j^n \rho_{j'}^n) = f_n^2, \quad \text{and} \quad E(\rho_j^n \rho_{j'}^{n'}) = f_n f_{n'}.$$

$$E(\delta_i^m \rho_i^m) = f_m^2, \qquad E(\delta_i^m \rho_i^{m'}) = f_m f_{m'},$$

$$E(\delta_i^m \rho_{i'}^m) = f_m^2, \quad \text{and} \quad E(\delta_i^m \rho_{i'}^{m'}) = f_m f_{m'}.$$

We may characterize any variate, $x_{ij}$, in terms of the population elements as follows:

$$x_{ij} = \sum_m \sum_n \delta_i^m \rho_j^n \tau_{mn},$$

which on substitution of

$$\tau_{mn} = \mu + G_m + G_n + S_{mn},$$

expands to

$$x_{ij} = \mu + \sum_m \delta_i^m G_m + \sum_n \rho_j^n G_n + \sum_m \sum_n \delta_i^m \rho_j^n S_{mn}.$$

This representation of $x_{ij}$ may be made in a more conventional form:

$$x_{ij} = \mu + l_i + t_j + (lt)_{ij},$$

where

$$l_i = \sum_m \delta_i^m G_m,$$

$$t_j = \sum_n \rho_j^n G_n,$$

and

$$(lt)_{ij} = \sum_m \sum_n \delta_i^m \rho_j^n S_{mn}.$$

We now consider the expected mean values and the expected square and cross product values of these various elements:

Expected mean values:

$$E(l_i) = \sum_m E(\delta_i^m) G_m = \sum_m f_m G_m = 0,$$

$$E(t_j) = \sum_n E(\rho_j^n) G_n = \sum_n f_n G_n = 0,$$

and

$$E\{(lt)_{ij}\} = \sum_m \sum_n E(\delta_i^m \rho_j^n) S_{mn} = \sum_m \sum_n f_m f_n S_{mn} = 0.$$

Parenthetically, we note that

$$E(x_{ij}) = \mu, \text{ for all } i \text{ and } j,$$

and

$$E(x_{..}) = \frac{E}{ab} X_{..} = \mu = \sum_m \sum_n f_m f_n \tau_{mn},$$

i.e. the sampling procedure generates the population about which inferences are to be made.

Expected values for square and cross products:

$$E(l_i^2) = E\{\sum_m \delta_i^m G_m\}^2$$

$$= \sum_m f_m G_m^2 = \sigma_{g.c.a.}^2, \text{ by definition.}$$

$$E(t_j^2) = E\{\sum_n \rho_j^n G_n\}^2$$

$$= \sum_n f_n G_n^2 = \sigma_{g.c.a.}^2, \text{ by definition.}$$

$$E\{(lt)_{ij}^2\} = E\{\sum_m\sum_n \delta_i^m \rho_j^n S_{mn}\}^2$$

$$= \sum_m\sum_n f_m f_n S_{mn}^2 = \sigma_{\text{s.c.a.}}^2 \text{ , by definition.}$$

$$E(l_i . l_{i'}) = E\{\sum_m \delta_i^m G_m\}.\{\sum_m \delta_{i'}^m G_m\}$$

$$= \sum_m\sum_{m'} f_m f_{m'} G_m G_{m'}$$

$$= (\sum_m f_m G_m).(\sum_{m'} f_{m'} G_{m'}) = 0.$$

Likewise,

$$E(t_j . t_{j'}) = 0.$$

$$E\{(lt)_{ij} . (lt)_{i'j}\} = E\{\sum_m\sum_n \delta_i^m \rho_j^n S_{mn}\}.\{\sum_m\sum_n \delta_{i'}^m \rho_j^n S_{mn}\}$$

$$= \sum_m\sum_n f_m^2 f_n S_{mn}^2 + \sum_{m\neq m'}\sum\sum_n f_m f_{m'} f_n S_{mn} S_{m'n}$$

$$= \sum_m\sum_n f_m f_n S_{mn}(\sum_q f_q S_{qn}) = 0.$$

$$E\{(lt)_{ij} . (lt)_{ij'}\} = E\{\sum_m\sum_n \delta_i^m \rho_j^n S_{mn}\}.\{\sum_m\sum_n \delta_i^m \rho_{j'}^n S_{mn}\}$$

$$= \sum_m\sum_n\sum_{n'} f_m f_n f_{n'} S_{mn} S_{mn'}$$

$$= \sum_m\sum_n f_m f_n S_{mn}(\sum_q f_q S_{mq}) = 0.$$

$$E\{(lt)_{ij} . (lt)_{i'j'}\} = E\{\sum_m\sum_n \delta_i^m \rho_j^n S_{mn}\}.\{\sum_m\sum_n \delta_{i'}^m \rho_{j'}^n S_{mn}\}$$

$$= \sum_m\sum_{m'}\sum_n\sum_{n'} f_m f_{m'} f_n f_{n'} S_{mn} S_{m'n'}$$

$$= (\sum_m\sum_n f_m f_n S_{mn}).(\sum_{m'}\sum_{n'} f_{m'} f_{n'} S_{m'n'}) = 0.$$

The expected values of cross products between elements of different classes are all zero. For example,

$$E(l_i . t_j) = E\{\sum_m \delta_i^m G_m\}.\{\sum_n \rho_j^n G_n\}$$

$$= \sum_m\sum_n f_m f_n G_m G_n$$

$$= (\sum_m f_m G_m).(\sum_n f_n G_n) = 0.$$

(ii) *Expectations of Mean Squares.*—When determining the expectations of mean squares we shall augment the genotypic model which was developed in the previous section to include the environmental effects, $e_{ijk}$, which are assumed to

be normally distributed and uncorrelated with the genotypic effects. Thus the appropriate model to consider is

$$x_{ijk} = \mu + l_i + t_j + (lt)_{ij} + e_{ijk} \qquad \begin{pmatrix} i = 1, \ldots, a \\ j = 1, \ldots, b \\ k = 1, \ldots, c \end{pmatrix},$$

where

$$E(l_i) = E(t_j) = E(lt)_{ij} = E(e_{ijk}) = 0,$$

$$E(l_i^2) = E(t_j^2) = \sigma_{\text{g.c.a.}}^2,$$

$$E(lt)_{ij}^2 = \sigma_{\text{s.c.a.}}^2,$$

$$E(e_{ijk}^2) = \sigma_e^2,$$

and the expected values for all cross products are zero.

The expectations of mean squares may be summarized as follows:

For $M_l$:

$$E(M_l) = E\left(\frac{1}{a-1}\right)\left\{\sum_i \frac{X_{i..}^2}{bc} - \frac{X_{...}^2}{abc}\right\}$$

$$= \sigma_e^2 + c\sigma_{\text{s.c.a.}}^2 + bc\sigma_{\text{g.c.a.}}^2.$$

For $M_t$:

$$E(M_t) = E\left(\frac{1}{b-1}\right)\left\{\sum_j \frac{X_{.j.}^2}{ac} - \frac{X_{...}^2}{abc}\right\}$$

$$= \sigma_e^2 + c\sigma_{\text{s.c.a.}}^2 + ac\sigma_{\text{g.c.a.}}^2.$$

For $M_{lt}$:

$$E(M_{lt}) = E\left\{\frac{1}{(a-1)(b-1)}\right\}\left\{\sum_i\sum_j \frac{X_{ij.}^2}{c} - \sum_i \frac{X_{i..}^2}{bc} - \sum_j \frac{X_{.j.}^2}{ac} + \frac{X_{...}^2}{abc}\right\}$$

$$= \sigma_e^2 + c\sigma_{\text{s.c.a.}}^2.$$

For $M_e$:

The sum of squares is obtained by subtraction. If we let $S_e$ be the error sum of squares, then we compute the mean square as

$$M_e = \left\{\frac{1}{ab(c-1)}\right\}S_e,$$

and

$$E(M_e) = \sigma_e^2.$$

Unbiased estimates of the population variances can now be obtained by equating the expected to the observed mean squares:

$$\hat{\sigma}_e^2 = M_e,$$

$$\hat{\sigma}_{\text{s.c.a.}}^2 = \frac{1}{c}(M_{lt} - M_e),$$

$$\hat{\sigma}^2_{\text{g.c.a.}} = \frac{1}{ac}(M_t - M_{lt}),$$

or

$$= \frac{1}{bc}(M_l - M_{lt}),$$

or a combination of these two estimators.

### (c) *Example 2.—Sampling Procedures Involving One Random Sample*

A single sample of $p$ inbreds is chosen at random with replacement from $S = I_1, I_2, \ldots, I_P$ with associated frequencies $F = f_1, f_2, \ldots, f_P$. The inbreds of the sample are designated arbitrarily as $I_1^*, I_2^*, \ldots, I_p^*$. All possible crosses are made among these lines. This procedure gives rise to a maximum of $p^2$ combinations which can be divided into three groups: (i) the $p$ parental lines themselves, i.e. those crosses resulting from $I_i^* \times I_i^*$; (ii) one set of $\frac{1}{2}p(p-1)$ $F_1$'s, i.e. those resulting from crossing $I_i^* \times I_j^*$ $(i<j)$; and (iii) the set of $\frac{1}{2}p(p-1)$ reciprocal $F_1$'s, i.e. those resulting from crossing $I_j^* \times I_i^*$ $(i<j)$.

Diallel crossing methods may vary depending upon whether the parental inbreds or the reciprocal $F_1$'s or both are included in the analysis. With this as a basis for classification there are four possible experimental methods: (1) parents, one set of $F_1$'s and reciprocal $F_1$'s are included (all $p^2$ combinations); (2) parents and one set of $F_1$'s are included but reciprocal $F_1$'s are not $\{\frac{1}{2}p(p+1)$ combinations$\}$; (3) one set of $F_1$'s and reciprocals are included but the parents are not $\{p(p-1)$ combinations$\}$; (4) one set of $F_1$'s but neither parents nor reciprocal $F_1$'s is included $\{\frac{1}{2}p(p-1)$ combinations$\}$.

The analyses for the four methods have been brought together by the author (Griffing 1956$b$) and hence will not be discussed at length here.

Theoretically, the important difference among these four methods is whether or not the parents are included. It was suggested by the author (Griffing 1956$a$) that the term "diallel" should be used for those methods which include the parents and the term "modified diallel" be used for methods 3 and 4 which do not.

It was shown (Griffing 1956$a$) that with the modified diallel methods, unbiased estimates of the population variances can be obtained from mean squares of the appropriate analyses of variance. Kempthorne (1956) derived the expectations of mean squares for diallel method 1 and showed that these mean squares alone could not yield unbiased estimates of the desired parameters.

### (i) *Diallel Method 1 (parents, one set of $F_1$'s and reciprocal $F_1$'s are included)*

In the analysis of variance which is given in Table 3, we assume a completely randomized design in which there are "$c$" observations for each parent and $F_1$. However, as before, in the construction of the model we consider only the genotypic effects of the parental and $F_1$ mean values. We denote these means as $x_{ii}$ and $x_{ij}$ $(i \neq j)$ respectively.

(1) *Construction of the model.*—We need to consider only one set of sampling variables:

$$\begin{cases} \delta_i^m = 1 \text{ if } I_m = I_i^* \\ \quad = 0 \text{ otherwise} \end{cases}.$$

The expected mean value of $\delta_i^m$ is

$$E(\delta_i^m) = f_m.$$

TABLE 3

ANALYSIS OF VARIANCE FOR DIALLEL METHOD 1

| Source | D.F. | Sums of Squares* | Mean Squares |
|--------|------|------------------|--------------|
| General combining ability | $p-1$ | $S_g$ | $M_g$ |
| Specific combining ability | $p(p-1)/2$ | $S_s$ | $M_s$ |
| Reciprocal effects | $p(p-1)/2$ | $S_r$ | $M_r$ |
| Error | $p^2(c-1)$ | $S_e$ | $M_e$ |

* Where

$$S_g = \frac{1}{2cp} \Sigma_i (X_{i..} + X_{.i.})^2 - \frac{2}{cp^2} X_{...}^2 ,$$

$$S_s = \frac{1}{2c} \Sigma_i \Sigma_j X_{ij.}(X_{ij.} + X_{ji.}) - \frac{1}{2cp} \Sigma_i (X_{i..} + X_{.i.})^2 + \frac{1}{cp^2} X_{...}^2 ,$$

$$S_r = \frac{1}{2c} \Sigma_{i<j} (X_{ij.} - X_{ji.})^2 .$$

The expected square and cross product values are

$$E(\delta_i^m \cdot \delta_i^m) = E(\delta_i^m) = f_m ,$$

$$E(\delta_i^m \cdot \delta_i^{m'}) = 0,$$

$$E(\delta_i^m \cdot \delta_{i'}^m) = f_m^2 ,$$

and

$$E(\delta_i^m \cdot \delta_{i'}^{m'}) = f_m f_{m'} .$$

The first difficulty is encountered when the variates are characterized in the form

$$x_{ij} = \Sigma\Sigma_{m\,n} \delta_i^m \delta_j^n \tau_{mn} .$$

The expected value of the parental variate, $x_{ii}$, is the mean of $\pi$, whereas the expected value of $x_{ij}$ $(i \neq j)$ is the mean of $\Pi$. In more formal terms we have

$$E(x_{ii}) = E\{\sum_m \sum_n \delta_i^m \delta_i^n \, \tau_{mn}\}$$

$$= \sum_m f_m \, \tau_{mm} \, ,$$

and

$$E(x_{ij}) = E\{\sum_m \sum_n \delta_i^m \delta_j^n \, \tau_{mn}\}$$

$$= \sum_m \sum_n f_m f_n \, \tau_{mn} \, ,$$

where $\sum_m f_m \, \tau_{mm}$ is the mean of $\pi$, the inbred population, and $\sum_m \sum_n f_m f_n \, \tau_{mn}$ is the mean of $\Pi$, the random mating population.

In a sense we may regard this diallel sampling method as generating a population which differs from the original random mating population, $\Pi$. The generated population is in fact a composite one which is made up of both $\pi$ and $\Pi$. The relative frequencies of the two sub-populations is dependent on the number of inbreds drawn in the sample, $p$. This is evident when considering the expectation of the sample mean.

$$E(x_{..}) = E(1/p^2)\{\sum_i x_{ii} + \sum_{i \neq j} \sum x_{ij}\}$$

$$= (1/p)\sum_m f_m \, \tau_{mm} + \{(p-1)/p\}\sum_m \sum_n f_m f_n \, \tau_{mn} \, .$$

In order to evaluate the expectations of mean squares for the analysis of variance associated with this diallel method, it is most convenient to consider two different mathematical models. One represents the sample variates $x_{ii}$ in terms of the parameters of $\pi$, and the other represents the sample variates $x_{ij}(i \neq j)$ in terms of the parameters of $\Pi$.

*Model for* $x_{ii}$

Let us describe the inbred population, define the parameters, and construct the mathematical model for $x_{ii}$.

As before, we characterize the inbred populations as the set of elements $S = I_1, I_2, \ldots, I_P$ with frequencies $f_1, f_2, \ldots, f_P$. Let

$$\mu_I = \sum_m f_m \, \tau_{mm}$$

be the population mean. The identity,

$$\tau_{mm} \equiv \mu_I + (\tau_{mm} - \mu_I)$$

exists, which may be written as

$$\tau_{mm} \equiv \mu_I + I_{mm} \, ,$$

where

$$\sum_m f_m \, I_{mm} = 0.$$

The total sum of squares is partitioned as

$$\sum_m f_m \, I_{mm}^2 = \mu_I^2 + \sum_m f_m \, I_{mm}^2 \, ,$$

and we denote

$$\sigma_I^2 = \sum_m f_m \, I_m^2$$

as the inbred population genotypic variance.

Turning to the genotypic components of the sample variates, we let

$$x_{ii} = \sum_m \delta_i^m \, \tau_{mm} = \mu_I + \sum_m \delta_i^m \, I_{mm} \, ,$$

which we rewrite in the model form as

$$x_{ii} = \mu_I + h_{ii} \, .$$

The properties of $h_{ii}$ are as follows:

$$E(h_{ii}) = E\{\sum_m \delta_i^m \, I_{mm}\} = \sum_m f_m \, I_{mm} = 0,$$

$$E(h_{ii}^2) = E\{\sum_m \delta_i^m \, I_{mm}\}^2 = \sum_m f_m \, I_{mm}^2 = \sigma_I^2 \, ,$$

and

$$\underset{(i \neq j)}{E(h_{ii}h_{jj})} = \sum_m \sum_n f_m f_n \, I_{mm} I_{nn} = \left(\sum_q f_q \, I_{qq}\right)^2 = 0.$$

*Model for* $x_{ij} \, (i \neq j)$

Let the representation of $x_{ij}$ be of the form

$$x_{ij} = \sum_m \sum_n \delta_i^m \, \delta_j^n \, \tau_{mn} \, ,$$

or on expanding

$$x_{ij} = \mu + \sum_m \delta_i^m \, G_m + \sum_n \delta_j^n G_n + \sum_m \sum_n \delta_i^m \, \delta_j^n \, S_{mn} \, ,$$

which in model form may be written as

$$x_{ij} = \mu + g_i + g_j + s_{ij} \, ,$$

where

$$g_i = \sum_m \delta_i^m \, G_m \, ,$$

$$g_j = \sum_n \delta_j^n \, G_n \, ,$$

and

$$s_{ij} = \sum_m \sum_n \delta_i^m \, \delta_j^n \, S_{mn} \, .$$

The properties of these elements are summarized as follows:
Expected mean values:

$$E(g_k) = E\{\underset{m}{\Sigma}\, \delta_k^m\, G_m\} = \underset{m}{\Sigma}\, f_m\, G_m = 0,$$

and

$$\underset{(i \neq j)}{E(s_{ij})} = E\{\underset{m\, n}{\Sigma\Sigma}\, \delta_i^m\, \delta_j^n\, S_{mn}\} = \underset{m\, n}{\Sigma\Sigma}\, f_m f_n\, S_{mn} = 0.$$

Expected values for square and cross products:

$$E(g_k^2) = E\{\underset{m}{\Sigma}\, \delta_k^m\, G_m\}^2 = \underset{m}{\Sigma}\, f_m\, G_m^2$$

$$= \sigma_{\text{g.c.a.}}^2\,, \text{ by definition.}$$

$$E(s_{ij}^2) = E\{\underset{m\, n}{\Sigma\Sigma}\, \delta_i^m \delta_j^n\, S_{mn}\}^2$$

$$= \underset{m\, n}{\Sigma\Sigma}\, f_m f_n\, S_{mn}^2$$

$$- \sigma_{\text{s.c.a.}}^2\,, \text{ by definition.}$$

$$E(g_k \cdot g_{k'}) = E\{\underset{m}{\Sigma}\, \delta_k^m\, G_m\}.\{\underset{m}{\Sigma}\, \delta_{k'}^n\, G_n\}$$

$$= \underset{m\, n}{\Sigma\Sigma}\, f_m f_n\, G_m G_n$$

$$= (\underset{m}{\Sigma}\, f_m\, G_m).(\underset{n}{\Sigma}\, f_n\, G_n) = 0.$$

$$E(s_{ij} \cdot s_{i'j}) = E\{\underset{m\, n}{\Sigma\Sigma}\, \delta_i^m \delta_j^n\, S_{mn}\}.\{\underset{m\, n}{\Sigma\Sigma}\, \delta_{i'}^m \delta_j^n\, S_{mn}\}$$

$$= \underset{m\, n}{\Sigma\Sigma}\, f_m f_n\, S_{mn}(\underset{q}{\Sigma}\, f_q\, S_{qn}) = 0.$$

$$E(s_{ij} \cdot s_{ij'}) = E\{\underset{m\, n}{\Sigma\Sigma}\, \delta_i^m \delta_j^n\, S_{mn}\}.\{\underset{m\, n}{\Sigma\Sigma}\, \delta_i^m \delta_{j'}^n\, S_{mn}\}$$

$$- \underset{m\, n}{\Sigma\Sigma}\, f_m f_n\, S_{mn}(\underset{q}{\Sigma}\, f_q\, S_{mq}) = 0.$$

$$E(s_{ij} \cdot s_{i'j'}) = E\{\underset{m\, n}{\Sigma\Sigma}\, \delta_i^m \delta_j^n\, S_{mn}\}.\{\underset{m\, n}{\Sigma\Sigma}\, \delta_{i'}^m \delta_{j'}^n\, S_{mn}\}$$

$$= \underset{m\, m'\, n\, n'}{\Sigma\,\Sigma\,\Sigma\,\Sigma}\, f_m f_{m'} f_n f_{n'}\, S_{mn} S_{m'n'}$$

$$= (\underset{m\, n}{\Sigma\Sigma}\, f_m f_n\, S_{mn}).(\underset{m'\, n'}{\Sigma\,\Sigma}\, f_{m'} f_{n'}\, S_{m'n'}) = 0.$$

Finally, before considering the expectations of mean squares, we need to examine the expectations of cross products of elements of one model with those of the other.

$$E(h_{ii} \cdot g_i) = E\{\underset{m}{\Sigma} \, \delta_i^m \, I_{mm}\} \cdot \{\underset{m}{\Sigma} \, \delta_i^m \, G_m\}$$

$$= \underset{m}{\Sigma} f_m \, G_m \, I_{mm} \, .$$

Let us denote this covariance as $\sigma_{gI}$ .

$$\underset{(i \neq j)}{E(h_{ii} \cdot g_j)} = E\{\underset{m}{\Sigma} \, \delta_i^m \, I_{mm}\} \cdot \{\underset{m}{\Sigma} \, \delta_j^m \, G_m\}$$

$$= \underset{m}{\Sigma} f_m \, G_m (\underset{n}{\Sigma} f_n \, I_{nn}) = 0.$$

$$\underset{(i \neq j)}{E(h_{ii} \cdot s_{ij})} = E\{\underset{m}{\Sigma} \, \delta_i^m \, I_{mm}\} \cdot \{\underset{m}{\Sigma}\underset{n}{\Sigma} \, \delta_i^m \delta_j^n \, S_{mn}\}$$

$$= \underset{m}{\Sigma} f_m \, I_{mm} (\underset{n}{\Sigma} f_n \, S_{mn}) = 0.$$

$$\underset{(i \neq j, \; i \neq k, \; j \neq k)}{E(h_{ii} \cdot s_{jk})} = E\{\underset{m}{\Sigma} \, \delta_i^m \, I_{mm}\} \cdot \{\underset{m}{\Sigma}\underset{n}{\Sigma} \, \delta_j^m \delta_k^n \, S_{mn}\}$$

$$= \underset{m}{\Sigma} f_m \, I_{mm} (\underset{m}{\Sigma}\underset{n}{\Sigma} f_m f_n \, S_{mn}) = 0.$$

(2) *Expectations of mean squares.*—In determining the expectations of mean squares, we shall add reciprocal as well as environmental effects to the genotypic models which were developed in the last section. The appropriate models are then

and $\qquad x_{iik} = \mu_I + h_{ii} + e_{iik} \, ,$

$$\underset{(i \neq j)}{x_{ijk}} = \mu + g_i + g_j + s_{ij} + r_{ij} + e_{ijk} \qquad \begin{Bmatrix} i \neq j = 1, \; \dots, \; p \\ k = 1, \; \dots, \; c \end{Bmatrix},$$

where

$$s_{ij} = s_{ji} \, , \qquad r_{ij} = -r_{ji} \, ,$$

$$E(h_{ii}) = E(g_k) = E(s_{ij}) = E(r_{ij}) = E(e_{ijk}) = 0,$$

$$E(h_{ii}^2) = \sigma_I^2, \qquad E(g_k^2) = \sigma_{g.c.a.}^2 \, , \qquad E(s_{ij}^2) = \sigma_{s.c.a}^2 \, , \qquad \cdot \cdot$$

$$E(r_{ij}^2) = \sigma_r^2 \, , \qquad E(e_{ijk}^2) = \sigma_e^2 \, ,$$

$$E(\mu_I \cdot \mu) = \mu_I \cdot \mu, \qquad E(h_{ii} \cdot g_i) = \sigma_{gI} \, ,$$

and the expectations of all other cross products are zero.

The expectations of mean squares are summarized below. These expectations were first given by Kempthorne (1956).

For $M_g$ :

$$E(M_g) = \left(\frac{1}{p-1}\right)\left\{\frac{1}{2cp}\ \Sigma(X_{i..}+X_{.i.})^2 - \frac{2}{cp^2}X_{...}^2\right\}$$

$$= \sigma_e^2 + c\left\{\frac{2}{p}\sigma_I^2 + \frac{2}{p}(p-2)^2\sigma_{g.c.a.}^2 + \frac{2}{p}(p-2)\sigma_{s.c.a.}^2 + \frac{4(p-2)}{p}\sigma_{gI}\right\}.$$

For $M_s$ :

$$E(M_s) = E\left\{\frac{2}{p(p-1)}\right\}\left\{\frac{1}{2c}\ \Sigma\Sigma\ X_{ij.}(X_{ij.}+X_{ji.}) - \frac{1}{2cp}\ \Sigma(X_{i..}+X_{.i.})^2 + \frac{1}{cp^2}X_{...}^2\right\}$$

$$= \sigma_e^2 + c\left\{\frac{2}{p}(\mu_I-\mu)^2 + \frac{2}{p^2}(p-1)\sigma_I^2 + \frac{8}{p^2}(p-1)\sigma_{g.c.a.}^2\right.$$

$$\left. + \frac{2}{p^2}(p^2-2p+2)\sigma_{s.c.a.}^2 - \frac{8}{p^2}(p-1)\sigma_{gI}\right\}.$$

For $M_r$ :

$$E(M_r) = E\left\{\frac{1}{p(p-1)}\right\}\left\{\frac{1}{c}\ \Sigma\Sigma(X_{ij.}-X_{ji.})^2\right\}$$

$$= \sigma_e^2 + 2c\sigma_r^2 .$$

For $M_e$ :

The error sum of squares may be obtained by subtraction.

$$E(M_e) = E\left(\frac{1}{p^2(c-1)}\right)S_e$$

$$= \sigma_e^2 .$$

These expectations are identical with those derived by Kempthorne (1956) except that his mean squares are on a mean basis whereas ours are on an individual basis, and he has further partitioned the reciprocal sum of squares. To equate the two derivations we need to substitute

$$\sigma_G^2 = 2\sigma_{g.c.a.}^2 + \sigma_{s.c.a.}^2 , \qquad \text{cov}(P, O) = \sigma_{g.c.a.}^2 , \qquad \text{and } C = \sigma_{gI}$$

in the equations of Kempthorne.

It is obvious that unbiased estimates of the population variance components cannot be estimated from these mean squares alone.

(ii) *Diallel Method 2 (parents and one set of $F_1$'s are included but reciprocal $F_1$'s are not)*

In the analysis of variance given in Table 4, we assume a completely randomized design in which there are "$c$" observations for each parent and $F_1$.

(1) *Construction of model.*—In constructing the genotypic models necessary for deriving the expectations of mean squares for this method, the arguments given for method 1 can be used. Thus the identical genotypic models are available.

(2) *Expectations of mean squares.*—We need to add only independent and normally distributed error effects to the genotypic models in order to construct a completely general model. The appropriate models are then:

$$x_{iik} = \mu_I + h_{ii} + e_{iik},$$

and

$$x_{ijk} \atop (i<j) = \mu + g_i + g_j + s_{ij} + e_{ijk} \qquad \left\{ \begin{matrix} i<j = 1, \ldots, p \\ k = 1, \ldots, c \end{matrix} \right\},$$

where

$$s_{ij} = s_{ji},$$

$$E(h_{ii}) = E(g_k) = E(s_{ij}) = E(e_{iik}) = E(e_{ijk}) = 0,$$

$$E(h_{ii}^2) = \sigma_I^2, \qquad E(g_k^2) = \sigma_{\text{g.c.a.}}^2, \qquad E(s_{ij}^2) = \sigma_{\text{s.c.a.}}^2, \qquad E(e_{iik}^2) = E(e_{ijk}^2) = \sigma_e^2,$$

$$E(\mu_I \cdot \mu) = \mu_I \cdot \mu, \qquad E(h_{ii} \cdot g_i) = \sigma_{gI},$$

and the expectations of all other cross products are zero.

TABLE 4

ANALYSIS OF VARIANCE FOR METHOD 2

| Source | D.F. | Sums of Squares* | Mean Squares |
|---|---|---|---|
| General combining ability | $p-1$ | $S_g$ | $M_g$ |
| Specific combining ability | $p(p-1)/2$ | $S_s$ | $M_s$ |
| Error | $p(p+1)(c-1)$ | $S_e$ | $M_e$ |

* Where

$$S_g = \frac{1}{c(p+2)} \left\{ \sum_i (X_{i..} + X_{ii.})^2 - \frac{4}{cp} X_{...}^2 \right\},$$

$$S_s = \frac{1}{c} \sum_{i \le j} \sum X_{ij.}^2 - \frac{1}{c(p+2)} \sum_i (X_{i..} + X_{ii.})^2 + \frac{2}{c(p+1)(p+2)} X_{...}^2.$$

The expectations of mean squares are summarized as follows:

For $M_g$ :

$$E(M_g) = E \left\{ \frac{1}{c(p-1)(p+2)} \right\} \cdot \left\{ \sum_i (X_{i..} + X_{ii.})^2 - \frac{4}{cp} X_{...}^2 \right\}$$

$$= \sigma_e^2 - c \left[ \left\{ \frac{p(p+1)}{(p-1)(p+2)} \right\} \{2\mu_I + (p-1)\mu\}^2 + \left\{ \frac{8}{(p-1)(p+2)} \right\} \sigma_I^2 \right.$$

$$\left. + \left\{ \frac{3p^2+4p-8}{p+2} \right\} \cdot \sigma_{\text{g.c.a.}}^2 + \left\{ \frac{p+4}{p+2} \right\} \sigma_{\text{s.c.a.}}^2 + \left\{ \frac{4(p+4)}{p+2} \right\} \sigma_{gI} \right].$$

For $M_s$ :

$$E(M_s) = E\left\{\frac{2}{p(p-1)}\right\} \cdot \left\{\frac{1}{c}\underset{i\leq j}{\Sigma\Sigma}X_{ij}^2 - \frac{1}{c(p+2)}\Sigma_i(X_{i..}+X_{ii.})^2 + \frac{2}{c(p+1)(p+2)}X_{...}^2\right\}$$

$$= \sigma_e^2 + c\left[\frac{2}{(p+1)}(\mu_I-\mu)^2 + \left\{\frac{2p}{(p+1)(p+2)}\right\}\sigma_I^2 + \left\{\frac{8p}{(p+1)(p+2)}\right\}\sigma_{g.c.a.}^2\right.$$

$$\left. + \left\{\frac{p^2+p+2}{(p+1)(p+2)}\right\}\sigma_{s.c.a.}^2 - \left\{\frac{8p}{(p+1)(p+2)}\right\}\sigma_{gI}\right].$$

For $M_e$ :

The error sum of squares may be obtained by subtraction.

$$E(M_e) = \left\{\frac{1}{(c-1)p(p+1)}\right\}S_e$$

$$= \sigma_e^2 .$$

It is clear that these mean squares cannot yield unbiased estimates of the $\Pi$ population variance. Thus we see that in both diallel methods 1 and 2, the mean squares involving parental as well as $F_1$ values have complex expectations which cannot be used for estimation purposes.

(iii) *Diallel Method 3.—A Modified Diallel Design (one set of $F_1$'s and reciprocals are included but the parents are not)*

In this modified diallel analysis we consider observations for all possible hybrid combinations resulting from the crosses $I_i^* \times I_j^*$ $(i\neq j)$. The appropriate analysis of variance for the completely randomized design in which there are "$c$" observations for each hybrid is given in Table 5.

(1) *Construction of model.*—If we characterize the hybrid genotypic mean value as

$$\underset{(i\neq j)}{x_{ij}} = \underset{m\,n}{\Sigma\Sigma}\delta_i^m\delta_j^n\,\tau_{mn} ,$$

we may note that

$$E(x_{ij}) = \underset{m\,n}{\Sigma\Sigma}f_m f_n\,\tau_{mn} = \tau_{..} ,$$

which is the mean of $\Pi$. Also,

$$E(x_{..}) = E\left\{\frac{1}{p(p-1)}\underset{i\neq j}{\Sigma\Sigma}x_{ij}\right\}$$

$$= \underset{m\,n}{\Sigma\Sigma}f_m f_n\,\tau_{mn} .$$

Thus the sampling procedure generates the population about which we wish to make inferences.

We are led, then, to the utilization of the model

$$x_{ij} = \mu + g_i + g_j + s_{ij}$$

to represent the genotypic mean value for the cross $L_i \times T_j$.  As before,

$$\mu = \tau_{..} ,$$

$$g_k = \sum_m \delta_k^m \, G_m ,$$

and

$$s_{ij} = \sum_m \sum_n \delta_i^m \, \delta_j^n \, S_{mn} = s_{ji} .$$

The properties of the elements are:

$$E(g_k) = E(s_{ij}) = 0,$$

$$E(g_k^2) = \sigma_{\text{g.c.a.}}^2, \qquad E(s_{ij}^2) = \sigma_{\text{s.c.a.}}^2 ,$$

and the expectations of all cross products are zero.

TABLE 5

ANALYSIS OF VARIANCE FOR "MODIFIED" DIALLEL METHOD 3

| Source | D.F. | Sums of Squares* | Mean Squares |
|---|---|---|---|
| General combining ability | $p-1$ | $S_g$ | $M_g$ |
| Specific combining ability | $p(p-3)/2$ | $S_s$ | $M_s$ |
| Reciprocal effects | $p(p-1)/2$ | $S_r$ | $M_r$ |
| Error | $p(p-1)(c-1)$ | $S_e$ | $M_e$ |

* Where

$$S_g = \frac{1}{2c(p-2)} \sum_i (X_{i..} + X_{.i.})^2 - \frac{2}{cp(p-2)} X_{...}^2 ,$$

$$S_s = \frac{1}{2c} \sum_{i<j} (X_{ij.} + X_{ji.})^2 - \frac{1}{2c(p-2)} \sum_i (X_{i..} + X_{.i.})^2 + \frac{1}{c(p-1)(p-2)} X_{...}^2 ,$$

$$S_r = \frac{1}{2c} \sum_{i<j} (X_{ij.} - X_{ji.})^2 .$$

(2) *Expectations of mean squares.*—In determining the expectations of mean squares for the completely randomized block analysis, we need augment the genotypic model with both reciprocal and environmental effects.  The appropriate model is:

$$x_{ijk} = \mu + g_i + g_j + s_{ij} + r_{ij} + e_{ijk} \qquad \begin{cases} i \neq j = 1, \ldots, p \\ k = 1, \ldots, c \end{cases} ,$$

where $\quad s_{ij} = s_{ji} , \qquad r_{ij} = -r_{ji} ,$

$$E(g_k) = E(s_{ij}) = E(r_{ij}) = E(e_{ijk}) = 0,$$

$$E(g_k^2) = \sigma^2_{\text{g.c.a.}}, \qquad E(s_{ij}^2) = \sigma^2_{\text{s.c.a.}}, \qquad E(r_{ij}^2) = \sigma_r^2,$$

$$E(e_{ijk}^2) = \sigma_e^2,$$

and the expectations of all other cross products are zero.

The expectations of mean squares are summarized below. Details of the derivations may be found in Kempthorne (1952).

For $M_g$ :

$$E(M_g) = E\left(\frac{1}{p-1}\right)\left\{\frac{1}{2c(p-2)}\sum_i(X_{i..}+X_{.i.})^2 - \frac{2}{cp(p-2)}X_{...}^2\right\}$$

$$= \sigma_e^2 + 2c\sigma^2_{\text{s.c.a.}} + 2c(p-2)\sigma^2_{\text{g.c.a.}}.$$

For $M_s$ :

$$E(M_s) = E\left\{\frac{2}{p(p-3)}\right\}\left\{\frac{1}{2c}\sum\sum_{i<j}(X_{ij.}+X_{ji.})^2 - \frac{1}{2c(p-2)}\sum_i(X_{i..}+X_{.i.})^2\right.$$

$$\left. + \frac{1}{c(p-1)(p-2)}X_{...}^2\right\}$$

$$= \sigma_e^2 + 2c\sigma^2_{\text{s.c.a.}}.$$

For $M_r$ :

$$E(M_r) = E\left\{\frac{1}{p(p-1)}\right\}\cdot\left\{\frac{1}{c}\sum\sum_{i<j}(X_{ij.}-X_{ji.})^2\right\}$$

$$= \sigma_e^2 + 2c\sigma_r^2.$$

For $M_e$ :

The error sum of squares may be obtained by subtraction.

$$E(M_e) = E\left\{\frac{1}{(c-1)p(p-1)}\right\}S_e$$

$$= \sigma_e^2.$$

We may estimate the variance components as follows:

$$\hat{\sigma}_e^2 = M_e,$$

$$\hat{\sigma}_r^2 = (M_r - M_e)/2c,$$

$$\hat{\sigma}^2_{\text{s.c.a.}} = (M_s - M_e)/2c,$$

and

$$\hat{\sigma}^2_{\text{g.c.a.}} = (M_g - M_s)/2c(p-2).$$

These estimates provide unbiased estimates of the population variances.

(iv) *Diallel Method 4.—A Modified Diallel Design (one set of $F_1$'s but neither parents nor reciprocal $F_1$'s are included)*

In this modified diallel analysis we consider observations for all hybrid combinations resulting from the crosses $I_i^* \times I_j^*$ $(i<j)$. The analysis of variance for the completely randomized design in which there are "$c$" observations for each hybrid combination is given in Table 6.

TABLE 6

ANALYSIS OF VARIANCE FOR "MODIFIED" DIALLEL METHOD 4

| Source | D.F. | Sums of Squares* | Mean Squares |
|---|---|---|---|
| General combining ability | $p-1$ | $S_g$ | $M_g$ |
| Specific combining ability | $p(p-3)/2$ | $S_s$ | $M_s$ |
| Error | $p(p-1)(c-1)/2$ | $S_e$ | $M_e$ |

* Where

$$S_g = \frac{1}{c(p-2)} \Sigma X_{i..}^2 - \frac{4}{cp(p-2)} X_{...}^2 ,$$

$$S_s = \frac{1}{c} \Sigma \Sigma_{i<j} X_{ij.}^2 - \frac{1}{c(p-2)} \Sigma_i X_{i..}^2 + \frac{1}{c(p-1)(p-2)} X_{...}^2 .$$

(1) *Construction of model.*—As in method 3 the genotypic effects of the cross $I_i^* \times I_j^*$ $(i<j)$ are represented by the model

$$x_{ij} = \mu + g_i + g_j + s_{ij} \qquad \left\{ \begin{array}{l} i<j = 1, \ldots, p \\ k = 1, \ldots, c \end{array} \right\},$$

where

$$E(\mu) = \tau_{..}, \qquad E(g_k) = E(\Sigma_m \delta_k^m G_m) = 0,$$

$$E(s_{ij}) = E(\Sigma \Sigma_{m\,n} \delta_i^m \delta_j^n S_{mn}) = 0,$$

$$E(g_k^2) = \Sigma_m f_m G_m^2 = \sigma_{\text{g.c.a.}}^2 , \qquad E(s_{ij}^2) = \Sigma \Sigma_{m\,n} f_m f_n S_{mn}^2 = \sigma_{\text{s.c.a.}}^2 ,$$

and the expectations of all cross products are zero.

(2) *Expectations of mean squares.*—The expectations of mean squares are summarized as follows:

For $M_g$ :

$$E(M_g) = E\left(\frac{1}{p-1}\right) \cdot \left\{\frac{1}{c(p-2)} \sum_i X_{i..}^2 - \frac{4}{cp(p-2)} X_{...}^2\right\}$$

$$= \sigma_e^2 + c\sigma_{\text{s.c.a.}}^2 + c(p-2)\sigma_{\text{g.c.a.}}^2 .$$

For $M_s$ :

$$E(M_s) = E\left\{\frac{2}{p(p-3)}\right\} \cdot \left\{\frac{1}{c} \sum_{i<j} X_{ij.}^2 - \frac{1}{c(p-2)} \sum_i X_{i..}^2 + \frac{1}{c(p-1)(p-2)} X_{...}^2\right\}$$

$$= \sigma_e^2 + c\sigma_{\text{s.c.a.}}^2 .$$

For $M_e$ :

The error sum of squares may be obtained by subtraction.

$$E(M_e) = E\left\{\frac{2}{(c-1)p(p-1)}\right\} S_e$$

$$= \sigma_e^2 .$$

The following estimators provide unbiased estimates of the population variance components.

$$\hat{\sigma}_e^2 = M_e ,$$

$$\hat{\sigma}_{\text{s.c.a.}}^2 = (M_s - M_e)/c,$$

and

$$\hat{\sigma}_{\text{g.c.a.}}^2 = (M_g - M_s)/c(p-2).$$

Thus it is apparent that unbiased estimates of the genotypic variances of the $\Pi$ population can be obtained from mean squares of either of the "modified" diallel crossing systems. The proof of this was given first by Griffing (1956a) in quite a different manner.

## III. Example 3.—An Example Involving Two Random Mating Populations

Consider the sampling situation in which samples are drawn from two different populations. Consider two random mating populations in equilibrium whose genotypic arrays can be represented by $\Pi_1$ and $\Pi_2$. By imposing an inbreeding system without selection on $\Pi_1$ and $\Pi_2$, the inbred populations $\pi_1$ and $\pi_2$ are derived. These inbred populations may be characterized by the sets $S_1 = {}_1I_1, {}_1I_2, \ldots, {}_1I_A$, with frequencies ${}_1f_1, {}_1f_2, \ldots, {}_1f_A$, and $S_2 = {}_2I_1, {}_2I_2, \ldots, {}_2I_B$ with frequencies ${}_2f_1, {}_2f_2, \ldots, {}_2f_B$, respectively. It is from these populations of inbreds that the sampling with replacement is made. From $S_1$, lines are randomly drawn with replacement to constitute the sample $L_1^*, \ldots, L_a^*$ and from $S_2$, lines are independently drawn with replacement to give the sample $T_1^*, \ldots, T_b^*$. All possible crosses are made among the $L^*$'s and $T^*$'s. These hybrid combinations provide the experimental material upon which observations are made. The question naturally arises,

can we estimate the genotypic variances, $\sigma^2_{g.c.a.}$ and $\sigma^2_{s.c.a.}$ of the populations $\Pi_1$ and $\Pi_2$ with the variance components, $\sigma^2_l$, $\sigma^2_t$, and $\sigma^2_{lt}$ ?

It is clear that the population about which inferences can be made is $\Pi_3 = \pi_1 \times \pi_2$. It can be easily shown that a mathematical model can be constructed, expectations of mean squares for the appropriate analysis of variance determined, and unbiased estimates made of the parameters of $\Pi_3$. However, these genotypic variances are not the same as those associated with $\Pi_1$ or $\Pi_2$, except in the case of $\Pi_1 = \Pi_2$. In this case the problem is the same as that in the first sampling problem of this study.

The following develops the above argument more rigorously:

### (a) Definition of Population Parameters

$\Pi_1$:

Let $_1\tau_{mn}$ represent the genotypic value of the hybrid $_1I_m \times _1I_n$. Then the following identity exists:

$$_1\tau_{mn} \equiv {}_1\mu + {}_1G_m + {}_1G_n + {}_1S_{mn} ,$$

where

$$_1\mu = {}_1\tau_{..} = \underset{m\,n}{\Sigma\Sigma} {}_1f_m \, {}_1f_n \, {}_1\tau_{mn} = \text{mean of } \Pi_1 ,$$

$$_1G_m = ({}_1\tau_{m.} - {}_1\tau_{..}) = (\Sigma {}_1f_n \, {}_1\tau_{mn} - \underset{m\,n}{\Sigma\Sigma} {}_1f_m \, {}_1f_n \, {}_1\tau_{mn})$$

$$= \text{g.c.a. effect for } m\text{th inbred},$$

$$_1G_n = ({}_1\tau_{.n} - {}_1\tau_{..}) = (\Sigma {}_1f_m \, {}_1\tau_{mn} - \underset{m\,n}{\Sigma\Sigma} {}_1f_m \, {}_1f_n \, {}_1\tau_{mn})$$

$$= \text{g.c.a. effect for } n\text{th inbred, and}$$

$$_1S_{mn} = ({}_1\tau_{mn} - {}_1\tau_{m.} - {}_1\tau_{.n} + {}_1\tau_{..})$$

$$= \text{s.c.a. effect associated with the hybrid } {}_1I_m \times {}_1I_n .$$

The following identity in sums of squares exists:

$$_1\sigma^2_G \equiv 2 \, {}_1\sigma^2_{g.c.a.} + {}_1\sigma^2_{s.c.a.} ,$$

where

$$_1\sigma^2_G = (\underset{m\,n}{\Sigma\Sigma} {}_1f_m \, {}_1f_n \, {}_1\tau^2_{mn} - {}_1\tau^2_{..}) = \text{total genotypic variance},$$

$$_1\sigma^2_{g.c.a.} = \underset{m}{\Sigma} {}_1f_m \, {}_1G^2_m = \text{g.c.a. variance},$$

and

$$_1\sigma^2_{s.c.a.} = \underset{m\,n}{\Sigma\Sigma} {}_1f_m \, {}_1f_n \, {}_1S^2_{mn} = \text{s.c.a. variance of } \Pi_1 .$$

$\Pi_2$:

The definitions for the parameters of $\Pi_2$ are similar to those given above for $\Pi_1$. We need replace the subscript 1 by 2, and, of course, remember that the summations involve $m, n = 1, \ldots, B$ rather than $m, n = 1, \ldots, A$ as with $\Pi_1$.

$\Pi_3 = \pi_1 \times \pi_2$:

Let $\xi_{mn}$ represent the genotypic value of the hybrid $_1I_m \times _2I_n$. Then the identity exists

$$\xi_{mn} \equiv \eta + L_m + T_n + (LT)_{mn},$$

where

$$\eta = \xi_{..} = \underset{m\,n}{\Sigma\Sigma}\ _1f_m\ _2f_n\ \xi_{mn} = \text{mean of } \Pi_3,$$

$$L_m = (\xi_{m.} - \xi_{..}) = (\underset{n}{\Sigma}\ _2f_n\ \xi_{mn} - \xi_{..})$$

$$= \text{g.c.a. effect of } m\text{th inbred of } \pi_1 \text{ when } \pi_2 \text{ is used as a tester population,}$$

$$T_n = (\xi_{.n} - \xi_{..}) = (\underset{m}{\Sigma}\ _1f_m\ \xi_{mn} - \xi_{..})$$

$$= \text{g.c.a. effect of } n\text{th inbred of } \pi_2, \text{ when } \pi_1 \text{ is used as a tester population,}$$

and

$$(LT)_{mn} = (\xi_{mn} - \xi_{m.} - \xi_{.n} + \xi_{..})$$

$$= \text{s.c.a. effect associated with the hybrid } _1I_m \times _2I_n, \text{ defined in population } \Pi_3.$$

The following identity in sums of squares exists:

$$\sigma_G^2 \equiv \sigma_l^2 + \sigma_t^2 + \sigma_{lt}^2,$$

where

$$\sigma_G^2 = (\underset{m\,n}{\Sigma\Sigma}\ _1f_m\ _2f_n\ \xi_{mn}^2 - \eta^2)$$

$$= \text{total genotypic variance of } \Pi_3,$$

$$\sigma_l^2 = \underset{m}{\Sigma}\ _1f_m\ L_m^2$$

$$= \text{g.c.a. variance among inbreds of } \pi_1, \text{ when } \pi_2 \text{ is used as tester population,}$$

$$\sigma_t^2 = \underset{n}{\Sigma}\ _2f_n\ T_n^2$$

$$= \text{g.c.a. variance among inbreds of } \pi_2, \text{ when } \pi_1 \text{ is used as a tester population,}$$

and

$$\sigma_{lt}^2 = \underset{m\,n}{\Sigma\Sigma}\ _1f_m\ _2f_n\ (LT)_{mn}^2$$

$$= \text{s.c.a. variance in } \Pi_3.$$

Note that $\sigma_l^2 \neq \sigma_t^2$.

## (b) Construction of Model

We now turn to the sample observations. As before we shall consider only the genotypic values in the construction of the model.

Let $x_{ij}$ represent the genotypic value of the hybrid $L_i^* \times T_j^*$. Following the arguments we have previously made regarding the use of dummy variables, we characterize $x_{ij}$ in terms of the elements of $\Pi_3$ as

$$x_{ij} = \sum_m \sum_n \delta_i^m \rho_j^n \xi_{mn} \, ,$$

which leads to the model

$$x_{ij} = \eta + l_i + t_j + (lt)_{ij} \, ,$$

where

$$\eta = \xi_{..} \, ,$$

$$l_i = \sum_m \delta_i^m L_m \, ,$$

$$t_j = \sum_n \rho_j^n T_n \, ,$$

and

$$(lt)_{ij} = \sum_m \sum_n \delta_i^m \rho_j^n (LT)_{mn} \, .$$

The properties of these elements are:

$$E(l_i) = E(t_j) = E\{(lt)_{ij}\} = 0,$$

$$E(l_i^2) = \sigma_l^2, \quad E(t_j^2) = \sigma_t^2, \quad E\{(lt)_{ij}^2\} = \sigma_{lt}^2 \, ,$$

and the expectations of all cross products are zero.

## (c) Expectations of Mean Squares

The expectations of mean squares are set out in Table 7. In this table we assume a completely randomized design with "$c$" observations for each hybrid combination. The genotypic model is, as usual, augmented by independent, normally distributed error terms.

It is clear that unbiased estimates of $\Pi_3$ population variances can be made as follows:

$$\hat{\sigma}_e^2 = M_e \, ,$$

$$\hat{\sigma}_{lt}^2 = (M_{lt} - M_e)/c,$$

$$\hat{\sigma}_t^2 = (M_t - M_{lt})/ac,$$

and

$$\hat{\sigma}_l^2 = (M_l - M_{lt})/bc.$$

Although unbiased estimates of variances of $\Pi_3$ can be obtained, these variances are not the g.c.a. and s.c.a. variances of $\Pi_1$ or $\Pi_2$. Thus $\sigma_l^2 \neq {}_1\sigma_{\text{g.c.a.}}^2$,

$\sigma_t^2 \neq {}_2\sigma_{\text{g.c.a.}}^2$, and $\sigma_{lt}^2 \neq {}_1\sigma_{\text{s.c.a.}}^2$ or ${}_2\sigma_{\text{s.c.a.}}^2$. To show, for example, that $\sigma_l^2 \neq {}_1\sigma_{\text{g.c.a.}}^2$, it is only necessary to show that $L_m \neq {}_1G_m$ since

$$\sigma_l^2 = \sum_m {}_1f_m L_m^2 \quad \text{and} \quad {}_1\sigma_{\text{g.c.a.}}^2 = \sum_m {}_1f_m {}_1G_m^2 .$$

Since
$$\dot{L}_m = (\xi_{m.} - \xi_{..})$$
$$= (\sum_n {}_2f_n \xi_{mn} - \sum_m\sum_n {}_1f_m {}_2f_n \xi_{mn}),$$

and
$$_1G_m = ({}_1\tau_{m.} - {}_1\tau_{..})$$
$$= (\sum_m {}_1f_n {}_1\tau_{mn} - \sum_m\sum_n {}_1f_m {}_1f_n {}_1\tau_{mn}),$$

$L_m \neq {}_1G_m$ unless ${}_1I_i \equiv {}_2I_i$, ${}_1f_i \equiv {}_2f_i$ for $i=1, \ldots, A$ and $A=B$, but, then, $\Pi_1 \equiv \Pi_2$ and the problem degenerates to the first sampling problem.

This illustrates the need for proper identification of the population about which inferences are to be made.

TABLE 7

ANALYSIS OF VARIANCE FOR EXAMPLE 3

| Source | D.F. | Mean Squares | Expectations of Mean Squares |
|---|---|---|---|
| Between $L$'s | $(a-1)$ | $M_l$ | $\sigma_e^2 + c\sigma_{lt}^2 + bc\sigma_l^2$ |
| Between $T$'s | $(b-1)$ | $M_t$ | $\sigma_e^2 + c\sigma_{lt}^2 + ac\sigma_t^2$ |
| $L \times T$ | $(a-1)(b-1)$ | $M_{lt}$ | $\sigma_e^2 + c\sigma_{lt}^2$ |
| Error | $ab(c-1)$ | $M_e$ | $\sigma_e^2$ |

## IV. Discussion

We are concerned with the estimation of general and specific combining ability variances of random mating populations. However, we are interested not so much in presenting a battery of experimental methods for estimating these variance components, as in presenting a logical basis for determining whether or not a proposed method yields unbiased estimates of the population variances.

The principal feature of this presentation is the use made of dummy variables in the construction of the mathematical models. Dummy variables have been elegantly used by Kempthorne (1952, 1957) and Wilk and Kempthorne (1955, 1956) in generalizing analysis of variance problems in which sampling is made *without* replacement from finite populations. In this study dummy variables are applied to analysis of variance problems in which sampling is made *with* replacement from finite populations. A complication arises in that the elements of the population are not assumed to have equal frequencies.

The notation of combining ability can be applied to any level of genetic organization, i.e. to genes, chromosomes, gametes, individuals, and even to groupings of individuals. We have chosen combining ability analyses involving inbred lines, which in effect are gamete combining ability analyses, because (i) the analyses are simple and this facilitates the presentation of the general argument, and (ii) considerable interest has been evidenced in the use of diallel crosses involving inbred lines, and thus a clarification of the variance estimation problem is worthwhile.

By using combining ability analyses of homozygous lines instead of the more usual analyses based on genes, a simpler interpretation is obtained. The genic analysis, although desirable, would be complicated and would detract from the main purpose of the paper.

The three examples have been chosen for the following reasons:

(1) The first example, in addition to providing a solution to a genetic problem which is of interest in itself, affords an excellent illustration of the use of dummy variables in problems of sampling with replacement from finite populations, the elements of which are not of equal frequency.

(2) The second example, or set of examples (i.e. the four diallel methods), provides a more difficult application of the principles involved. Also, from the point of view of genetical interest, this presentation brings together the general solutions for all four methods.

(3) The third example is a generalization of the first example. It clearly indicates the necessity of exactly identifying the populations about which inferences are to be made. Thus, although sampling is carried out in populations $\pi_1$ and $\pi_2$, inferences cannot be made about the variances of population $\Pi_1$ or $\Pi_2$.

This paper is presented as part of an overall study of the concept of general and specific combining ability as applied to plant and animal breeding. In future studies it is hoped to present a more comprehensive treatment of the use of the dummy variables in the estimation of combining ability effects and variances.

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# STUDIES OF DORMANCY IN THE SEEDS OF SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM* L.)

## I. BREAKING OF DORMANCY BY CARBON DIOXIDE AND BY ACTIVATED CARBON

By L. A. T. Ballard*

### Summary

Two new methods for breaking the dormancy of subterranean clover (*Trifolium subterraneum* L.) seeds are described, viz. treatment of imbibed seeds with a low concentration of carbon dioxide, and with activated carbons.

Responses to these treatments are found widely throughout the species.

Between approximately 0·5 and 5 per cent. carbon dioxide no differences in response occur.

The activated carbons do not appear to adsorb inhibitors, as is their usual action, and the evidence is consistent with the supposition that they produce carbon dioxide which then initiates germination.

The relevance of these findings to some aspects of seed testing and field practice is indicated.

## I. Introduction

It is well known that, in common with those of many other leguminous species, the seeds of subterranean clover (*Trifolium subterraneum* L.) may either be hard, or possess a post-harvest dormancy of shorter or longer duration, or both. The delay in germination occasioned by either of these features has considerable agronomic significance, and information on the nature and origin of these conditions, as well as on mechanisms responsible for release from them should be of value.

Aitken (1938) established the morphological basis of hardness of seed, as well as the influence of conditions during seed maturation on the development of this character, and Loftus Hills (1942, 1944a, 1944b, 1944c, 1944d) has contributed most to knowledge of dormancy in this species. He showed that dormancy was a varietal character, and listed the relative dormancy for most of the strains then current, as well as the times necessary for passage out of dormancy. Delayed harvest was found to reduce the proportion of dormant seed, and an earlier finding of Woodforde (1935), that exposure of imbibed seed to low temperature accelerates release from dormancy, was confirmed. Loftus Hills also noted that removal of the testa had a similar effect.

The present work is concerned solely with dormancy and in this paper two further, and highly effective, methods of breaking dormancy will be described.

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## II. Methods

Seed was obtained from spaced plants field-grown at Canberra. At commercial ripeness, tops and burrs were harvested, dried at a temperature not exceeding 30°C, and threshed on a light, machine-operated rubber thresher. Seed was stored in the laboratory in closed (but not sealed) containers which were frequently opened during the course of the investigation.

In order to avoid the complication of any residual hard-seededness, all seeds were subjected either to percussion or to light scarification at the time of test. Such seeds were immersed in a shallow layer of boiled mains water for 1–2 hr, and during the subsequent 1–4 hr only swollen or swelling seeds were taken into germination trials.

Standard or control conditions were provided by setting out replicates of 50 or 25 (rarely) seeds in 10-cm diameter petri dishes on two 9-cm circles of filter paper moistened with 4 ml of boiled mains water.

Gas treatments were applied in two ways. In the first, replicates of 30–50 seeds were placed on moistened filter paper in tubes of approximately 45 ml capacity, through which gases of the required composition were passed at the rate of 10–20 ml per minute. The gas mixtures were obtained from an apparatus of the type described by Bailey (1954). When carbon dioxide content was varied, oxygen was maintained constant at 21 per cent.,* the deficit being supplied by nitrogen. When only oxygen content was varied, nitrogen was appropriately adjusted. In the second method, replicates of 50 or 25 (usually) seeds were placed on moistened filter paper in flat-sided bottles of approximately 160 ml capacity. Each bottle was perfused, sufficiently to ensure 5–10 exchanges, with a gas mixture of the required composition, and then sealed by a washer and screw cap. Alternatively, an amount of gas necessary to give the required concentration was displaced into a bottle which was then sealed. When carbon dioxide was so added concomitant decreases in oxygen and nitrogen concentrations ensued. It is appreciated that respiratory and other processes would cause changes in the concentrations thus established; but it will later be seen that these are unimportant in relation to the magnitude of the effects produced by the initial concentrations of the gases.

For tests involving the use of adsorbents, replicates of 25 seeds were placed in 10-cm diameter petri dishes in the usual way, and each seed was covered with a small, standard scoopful of the particular adsorbent. The weight of adsorbent applied per seed varied with the nature of the adsorbent—for the activated carbons (the most important class investigated) it was of the order of 0·02 g.

Unless otherwise stated all containers were held in the dark at $22\pm1$°C and examined in either diffuse daylight or weak fluorescent light at approximately 24-hr intervals, for at least so long as germinations were frequent. Seeds whose radicles showed positive geotropism were counted as germinated and removed, except from sealed containers. For the latter, daily germination counts accurate to $\pm1$ were made through the walls, and precise counts were made at the termination of the experiment.

*The composition of gas mixtures is stated throughout as per cent. by volume.

For many of the results presented the differences between treatments are readily apparent, and only estimates of variability between replicates are given. Where it was desirable to carry out analyses of variance it was necessary to transform the percentage values. The angular transformation was employed.

## III. Results

### (a) The Time Course of Germination in Dormant Samples

Most of the results to be presented have been obtained with the highly dormant strain Burnerang, and with Mt. Barker which shows moderate dormancy. As a background to these results it is desirable to present information on the germination patterns found in such dormant varieties.
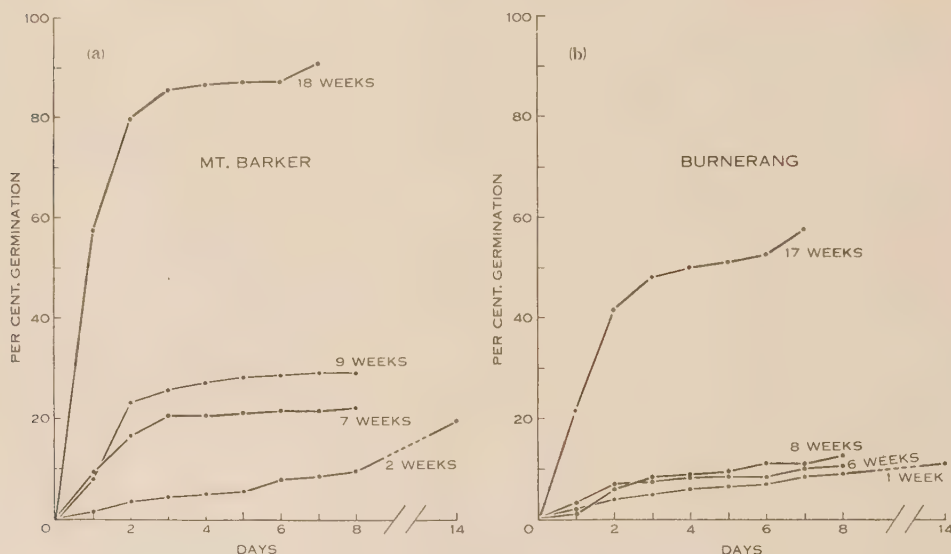


Fig. 1.—The time course of germination. (a) Mt. Barker strain, 2, 7, 9, and 18 weeks after harvest; (b) Burnerang strain, 1, 6, 8, and 17 weeks after harvest.

Both the daily germination during any one test, and the general level of germination at different points in storage life show interesting changes. From Figure 1 it may be seen that germination in both strains is low initially and increases with increasing rapidity. The initial rate of increase is greater in Mt. Barker than Burnerang. For the later tests it also seems that, under the conditions employed here, asymptotic germination values are reached in 7–8 days or less; but longer periods are required in the earliest test.

It seemed probable that, in any seed sample, rate of germination and absolute germinability should be positively correlated, and this supposition has been further examined.

Estimates of rate have been obtained by calculating the rate index according to Bartlett (1937). This is a somewhat arbitrary measure which takes into account the numbers of seeds which have germinated at successive observations, in relation

to the final number germinated. In effect, it measures the rate of germination of those seeds which actually germinate. Another estimate of rate has been obtained by finding the time required for a sample to reach 50 per cent. of its asymptotic germination. These values were derived, by interpolation, from the time-course curves of individual replicates. Where a true asymptote was not reached the final value recorded was used for this purpose. Both methods of estimating rate are relatively insensitive, especially when germination is low, and the consequent variability in small samples is high.

These data are collected in Table 1, and trends in the expected direction are present on both rate bases. For Mt. Barker, the difference between the rate index initially and the rate index for the three later storage points taken together is significant ($P < 0.05$); but the smaller difference is not significant for Burnerang.

TABLE 1

AMOUNT AND RATE OF GERMINATION AT INTERVALS FROM HARVEST

Germination and rate index at day 7 of test

| Strain | Time from Harvest (weeks) | Germination (%) | Rate Index | Time to 50% of Final or Asymptotic Value (days) |
|---|---|---|---|---|
| Mt. Barker | 2 | $8.5 \pm 1.0$ | $0.629 \pm 0.105$ | $8.1 \pm 1.39$ |
| | 7 | $21.5 \pm 2.4$ | $0.876 \pm 0.038$ | $1.3 \pm 0.27$ |
| | 9 | $29.0 \pm 4.7$ | $0.829 \pm 0.004$ | $1.4 \pm 0.06$ |
| | 18 | $90.5 \pm 1.7$ | $0.905 \pm 0.025$ | $0.8 \pm 0$ |
| Burnerang | 1 | $8.5 \pm 2.6$ | $0.706 \pm 0.072$ | $3.0 \pm 0.79$ |
| | 6 | $10.0 \pm 2.4$ | $0.777 \pm 0.041$ | $2.0 \pm 0.52$ |
| | 8 | $11.5 \pm 3.0$ | $0.622 \pm 0.118$ | $3.0 \pm 0.86$ |
| | 17 | $57.5 \pm 2.6$ | $0.802 \pm 0.037$ | $1.3 \pm 0.15$ |

It is clear that, in this instance, any relation between rate index and per cent. germination would be markedly curvilinear, with the curve concave to the germination axis.

Another set of data is presented in Figure 2. Here, at one storage point, germination was raised to different levels by different dormancy-breaking treatments, and the correlation between treatment per cent. germination and treatment rate index is 0.959 ($P < 0.05$). In this case the relationship is linear over the range of values obtained.

It should be recorded that, in the several sets of data available for analysis, significance was not invariably achieved in the apparent trend, and the causes of such variation are not known. A relationship appears to be better established when comparisons are made within one strain for one trial, rather than between strains and trials. Moreover, the precise form of such a relationship is variable, and it is likely that this is a reflection of differences in post-harvest maturation in relation to the exact actions of the dormancy-relieving agents.

Further work would be necessary to clarify these features, and for the present it is warranted to conclude only that the occurrence of a higher rate index may be taken as supporting evidence of a reduction in dormancy status.

### (b) Carbon Dioxide and Germination

(i) *The Effect.*—Results of preliminary experiments suggested that an increased partial pressure of carbon dioxide facilitated the germination of dormant seeds, and this was substantiated in direct tests.
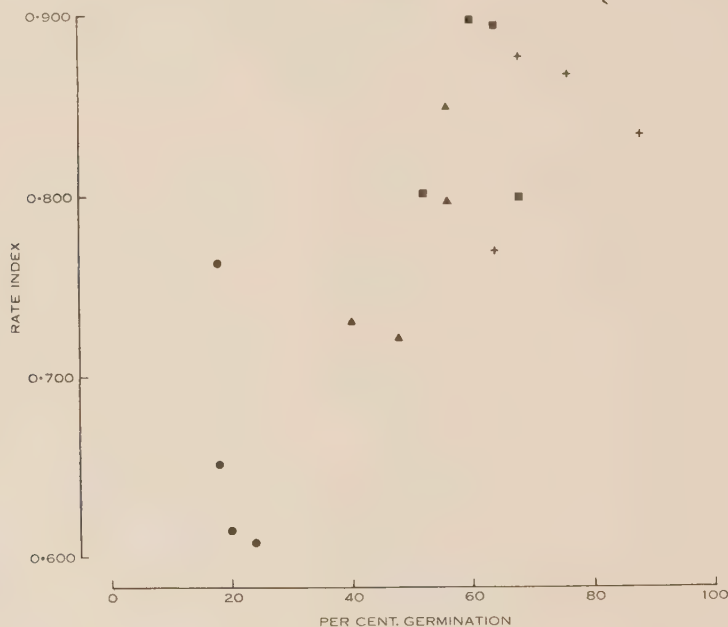


Fig. 2.—Relation between per cent. germination and rate index, Mt. Barker strain, following control treatment ( ● ) and treatment with three different activated carbons ( ▲, ■, + ).

In these experiments, in order to avoid any ambiguity which may have been introduced if volatile metabolites (in addition to respiratory carbon dioxide) were produced by the seeds, tests were conducted in gas streams of the required composition.

The results of one typical experiment are presented in Table 2. In this experiment, seeds were first exposed to standard germination conditions, and those which germinated were removed daily for 8 days, at which time germination had virtually ceased. The remaining, i.e. most dormant, seeds were randomized between control and carbon dioxide treatments. The removal of the readily germinable seeds accentuated treatment effects. Practically no further germination occurred when the seeds were exposed to air, either still (dishes) or moving (tubes); but nearly all seeds exposed to carbon dioxide promptly germinated.

Essentially similar results were obtained in four other experiments, in which the seeds were exposed, with or without the pre-incubation period, to air streams enriched to contain from 0·3–4·5 per cent. carbon dioxide. In one experiment the carbon dioxide was derived from A.R. reagents.

This simple type of experiment provides unequivocal evidence that carbon dioxide is an agent which initiates germination.

The marked response to carbon dioxide in so low a concentration as 0·3 per cent. suggested the possibility that the respiratory carbon dioxide evolved by dormant seeds, if allowed to accumulate in sealed vessels, could itself initiate germination. This proved to be so (e.g. per cent. germinations for Mt. Barker on day 2 of test: open vessels, 46 ±8; sealed vessels, 96±2). This effect is usually enhanced

TABLE 2

EFFECT OF CARBON DIOXIDE ON THE GERMINATION OF DORMANT SEEDS

Values are cumulative per cent. germinations. Mt. Barker strain. Preliminary incubation period 8 days, during which 21 per cent. germinated. Carbon dioxide concentration 0·3–0·4 per cent.

| Day | Tube 1 | | Tube 2 | | Tube 3 | | Tube 4 | | Control Dishes | |
| | Gas | Germination (%) | Gas | Germination (%) | Gas | Germination (%) | Gas | Germination (%) | 1 | 2 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Air | 0 | Air | 0 | $CO_2$ | 92 | $CO_2$ | 86 | 0 | 0 |
| 2 | Air | 0 | Air | 0 | $CO_2$ | 92 | $CO_2$ | 91 | 0 | 0 |
| 3 | Air | 0 | Air | 0 | $CO_2$ | 96 | $CO_2$ | 100 | 0 | 0 |
| 4 | Air | 0 | Air | 0 | $CO_2$ | 96 | $CO_2$ | 100 | 4 | 0 |
| 5 | Air | 4 | $CO_2$ | 71 | | | | | 4 | 0 |
| 6 | Air | 4 | $CO_2$ | 81 | | | | | 4 | 0 |
| 7 | Air | 4 | $CO_2$ | 86 | | | | | 4 | 0 |

by the presence in the germination sample of one or more non-dormant seeds. Such seeds germinate promptly, and the carbon dioxide output of the seedling rises sharply, with the consequent rapid initiation of germination of the remainder. However, even when the non-dormant seeds are excluded by pre-incubation, as described above, a similar result is obtained after a somewhat longer time lag.

(ii) *Quantitative Aspects.*—This autocatalytic action of respiratory carbon dioxide indicates that investigation of quantitative aspects should be made in gas streams. However, as appropriate facilities were not available, approximate data were obtained by exposing seeds in sealed bottles to atmospheres of known initial carbon dioxide content.

Some estimate of the drift from these initial concentrations may be made. If the respiratory output of carbon dioxide is taken to be 1 $\mu$l/hr/seed (a value in fair agreement with that calculable from the data of Black (1955) on dry weight losses during germination of subterranean clover, and also with that reported by Stiles

and Leach (1932) for the respiration of *Lathyrus odoratus* L. early in germination), the initial per cent. compositions in the present experiments would rise daily by 0·4 units.

TABLE 3

RELATION BETWEEN GERMINATION AND INITIAL CONCENTRATION OF CARBON
DIOXIDE

| Initial CO$_2$ Concn. (%) | Cumulative Germination (%) | | |
|---|---|---|---|
| | Day 1 | Day 3 | Day 6 |
| Atmospheric* | 0 ± 0 | 4 ± 4 | 5 ± 5 |
| 2·5 | 8 ± 4 | 94 ± 2 | 98 ± 2 |
| 5 | 4 ± 4 | 86 ± 10 | 94 ± 2 |
| 10 | 2 ± 2 | 74 ± 2 | 84 ± 4 |

*Not sealed.

From Table 3 it is seen that the main treatment effects are apparent before gross changes in carbon dioxide concentration would have occurred. Only when the initial concentration exceeds 5 per cent. do inhibitory effects occur, and between this value and 0·3 per cent. (the lowest concentration investigated by the gas stream method) a broad optimum exists. The standard carbon dioxide concentration adopted for later experiments was exposure to an atmosphere initially containing 2·5 per cent. carbon dioxide.

TABLE 4

EFFECT ON VIABILITY AND DORMANCY OF EXPOSURE TO 100 PER CENT.
CARBON DIOXIDE

| Duration of Exposure (days) | Germination during Exposure (%) | After Transfer to Air | | |
|---|---|---|---|---|
| | | Immediately Germinable (%) | Dormant (%) | Not Viable (%) |
| 1 | 0 | 67 | 26 | 7 |
| 4 | 0 | 73 | 3 | 24 |
| 8 | 0 | 31* | 0 | 69 |

*Many abnormals.

As would be expected, very high concentrations of carbon dioxide inhibit germination. However, Table 4 shows that a surprisingly high percentage of seeds retain viability for as long as 4 days' exposure to 100 per cent. carbon dioxide. There is evidence that shorter exposures induce some secondary dormancy.

A further finding is that reduction of oxygen tension does not interfere with the stimulation of germination induced by carbon dioxide in the optimal range,

until the partial pressure of oxygen falls to approximately half that of the normal atmosphere.

<center>(c) <i>Activated Carbon and Germination</i></center>

(i) *The Effect*

Treatment of dormant seed with activated carbons also brings about rapid and high germination.

Of approximately 20 carbon samples tested, all have given some response (with the possible exception of No. 2 of Table 5). The numbered carbons of Table 5 were prepared from a uniform, chemically pure sample by heat activation at different

<center>TABLE 5</center>

<center>EFFECT OF ACTIVATED CARBONS ON AMOUNT AND RATE OF GERMINATION</center>
<center>Germination and rate index at day 7 of test. Mt. Barker strain</center>

| Activated Carbon No. | Germination (%) | Means of Transformed Per Cent. Values | Rate Index |
|---|---|---|---|
| Nil | 46·7 | 43·1 | 0·480 |
| "Norit FNX" | 93·3 | 77·8 | 0·855 |
| 1 | 84·0 | 67·6 | 0·643 |
| 2 | 42·7 | 40·4 | 0·678 |
| 3 | 73·3 | 59·6 | 0·704 |
| 4 | 89·3 | 71·0 | 0·729 |
| 5 | 86·7 | 69·3 | 0·704 |
| 6 | 93·3 | 75·2 | 0·808 |
| 7 | 88·0 | 69·9 | 0·755 |
| 8 | 93·3 | 75·2 | 0·701 |
| 9 | 86·7 | 68·9 | 0·667 |
| 10 | 82·7 | 67·1 | 0·772 |
| Least significant difference $(P < 0.05)$ | | 14·0 | 0·075 |
| „        „        „      $(P < 0.01)$ | | 19·0 | 0·094 |

temperatures under nitrogen, to give a series with characteristics ranging from acidic (No. 1) to alkaline (No. 10). Taken as a whole, the carbons markedly increased both the amount of final germination (although at the time of test the control germination was substantial), and also the rate of germination. In this case differences between the various carbons scarcely achieved significance. However, significant differences have been observed between other samples of carbons, tested on other occasions, e.g. those of Figure 2. It is not known whether these two types of response represent real differences between the carbons themselves, or are caused by differences in the dormancy status of the seeds.

An indication of the rapidity with which the carbons initiate germination is the shortness of time they require to be in contact with seeds. The data of Table 6 were obtained in an experiment in which seeds were first covered with carbon in the usual manner, and at intervals replicates were washed free of carbon, and thereafter

treated as the controls. A significant increase in germination results from as little as 4 hours' contact.

(ii) *Mechanism*

While the wide applicability and high efficiency of this treatment in overcoming dormancy is securely founded, the mechanism by which the result is achieved is less surely known. The most probable actions of carbons are firstly, removal of inhibitor(s) either by adsorption or destruction, and secondly, production of carbon dioxide. The evidence for such actions may now be examined.

TABLE 6

EFFECT OF DURATION OF CONTACT OF ACTIVATED CARBON WITH SEEDS ON DORMANCY BREAKING

Values are mean cumulative per cent. germinations. Activated carbon, "Norit FNX". Mt. Barker strain

| Contact Time (hr) | Day 3 | | Day 6 | |
|---|---|---|---|---|
| | Germination (%) | Means of Transformed Per Cent. Values | Germination (%) | Means of Transformed Per Cent. Values |
| 0 | 12 | 19·1 | 20 | 26·1 |
| 1 | 16 | 23·4 | 24 | 29·3 |
| 2 | 16 | 23·4 | 16 | 23·4 |
| 4 | 40 | 39·3 | 50 | 45·0 |
| 6 | 52 | 46·2 | 58 | 49·8 |
| Permanent | 90 | 71·7 | 98 | 84·3 |
| Least significant difference ($P<0.05$) | | 16·4 | | 15·0 |
| „  „  „  ($P<0.01$) | | 24·9 | | 22·7 |

(1) *Inhibitor Adsorption.*—This is usually taken to be the action of activated carbons when they are effective in promoting seed germination. If this were so in the present case, it might reasonably be expected that other adsorbents could be found with at least qualitatively, even if not quantitatively, similar actions. However, no such adsorbent has been found among those tested.

The results of such trials are not presented in detail. Some adsorbents corresponding to Figure 3, curve 1, had a deleterious effect on germination (e.g. magnesium trisilicate, Fuller's earth "Fulmont", Fuller's earth "Tonsil AC"); others, Figure 3, curve 2, were relatively indifferent (e.g. bentonite, kieselguhr, aluminium hydroxide). Another group, Figure 3, curve 3, improved germination, some to a marked extent (e.g calcium carbonate, "Cellite", cellulose powder, activated alumina). However, it will be noted that this action arose after a marked lag phase, in sharp distinction to the action of activated carbons and carbon dioxide. It is suggested that the adsorbents of this group are merely more effective in retarding the diffusion of respiratory carbon dioxide away from the seeds, the germinations of which are thus initiated in a manner akin to sealing in a germination vessel.

(2) *Destruction of Inhibitors.*—Garten and Weiss (1955) have pointed out that some activated carbons produce peroxides, and it may be that these are then able destructively to oxidize inhibitors. Table 7 shows that there is, in fact, a promotive effect by hydrogen peroxide. However, this effect is seen only at very high concentrations which could scarcely correspond to the equivalent concentrations found in carbons. At such concentrations there is no effect. The high concentrations were observed to loosen the testas, and it seems that the hydrogen peroxide has acted chemically only to produce the result of mechanical removal or loosening observed by Loftus Hills (1944*a*).
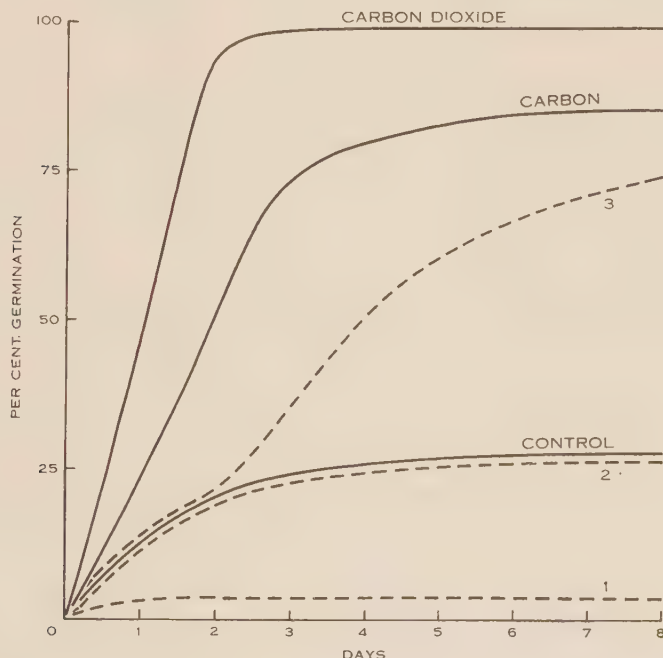


Fig. 3.—Schematic representation of the time course of germination of dormant seeds treated with carbon dioxide, activated carbon, and other classes of adsorbents (curves 1, 2, and 3).

(3) *Production of Carbon Dioxide.*—Only inferential evidence is available to support this hypothesis—that only carbons produce the effect is indeed suggestive.

However, activated carbon, previously wetted and allowed to equilibrate, is no less effective in promoting germination than carbon applied dry, and hence becoming wetted around the seeds (e.g. per cent. germinations for Mt. Barker on day 3 of test: control, $44\pm8$; dry carbon, $76\pm4$; wetted carbon, $72\pm12$). This means that any carbon dioxide which could be evolved by the activated carbon does not arise by displacement of previously adsorbed carbon dioxide, and presumably must arise by direct oxidation. Such a reaction can occur, though at the relatively low temperatures of physiological experiments the rate would be low (see Jones and Townend (1946) for a discussion on the formation of a carbon-water

oxygen complex, and the conditions determining its breakdown to carbon monoxide and carbon dioxide in differing proportions).

TABLE 7

EFFECT OF HYDROGEN PEROXIDE ON GERMINATION

Values are cumulative per cent. germinations. Mt. Barker strain

| Concentration (M) | Day 2 | Day 6 |
|---|---|---|
| 0 | $6 \pm 6$ | $16 \pm 4$ |
| 3 | $0 \pm 0$ | $0 \pm 0*$ |
| $6 \times 10^{-1}$ | $62 \pm 6$ | $92 \pm 0$ |
| $1 \cdot 2 \times 10^{-1}$ | $18 \pm 2$ | $52 \pm 16$ |
| $2 \cdot 4 \times 10^{-2}$ | $2 \pm 2$ | $20 \pm 4$ |
| $4 \cdot 8 \times 10^{-3}$ | $0 \pm 0$ | $10 \pm 2$ |
| $9 \cdot 6 \times 10^{-4}$ | $12 \pm 8$ | $28 \pm 8$ |

*Seeds killed.

It was thought that information might be obtained by incubating seeds in the presence of alkali, which might be expected to absorb carbon dioxide, respiratory and

TABLE 8

GERMINATION RESPONSES OF STRAINS TO CARBON DIOXIDE, ACTIVATED CARBON, OR LOW TEMPERATURE

Values are mean per cent. germinations. Initial carbon dioxide concentration, 2·5 per cent.; activated carbon, "Norit A"; low temperature treatment, 1 day at 10°C

| Strain | Day 3 | | | | Day 6 | | |
|---|---|---|---|---|---|---|---|
| | Control | $CO_2$ | Carbon | Cold | Control | Carbon | Cold |
| Portugal, C.P.I. 19465* | 12 | 93 | 63 | 29 | 14 | 75 | 30 |
| Algiers, C.P.I. 19455 | 4 | 98 | 69 | 40 | 26 | 73 | 66· |
| Cyprus, C.P.I. 19448 | 20 | 100 | 54 | 53 | 32 | 76 | 67 |
| Turkey, C.P.I. 15077A | 30 | 96 | 71 | 75 | 38 | 75 | 84 |
| Burnerang | 35 | 100 | 90 | 77 | 38 | 96 | 79 |
| Canary Is. | 34 | 94 | 77 | 75 | 46 | 81 | 80 |
| Northam First Early | 42 | 99 | 55 | 48 | 58 | 65 | 78 |
| Morocco, C.P.I. 19458 | 53 | 100 | 90 | 98 | 65 | 94 | 98 |
| Greece, C.P.I. 19479B | 65 | 100 | 51 | 84 | 70 | 69 | 89 |
| Daliak | 58 | 100 | 65 | 90 | 72 | 74 | 93 |
| Portugal, C.P.I. 19472 | 78 | 100 | 89 | 95 | 80 | 94 | 96 |

*Commonwealth Plant Introduction number.

otherwise, and so nullify or reduce the effects of sealing and application of carbon. Experiments to investigate this were conducted in petri dishes fitted, after the

fashion of Conway vessels, with central wells, which contained either alkali or water. Seeds were arranged in the annular space and could be covered with carbon. The dishes were either merely covered (open), or provided with sealed cover glasses. In some experiments all possible treatment combinations were employed.

The data are too voluminous for presentation; but the following more important results can be stated: (1) Sealing always accelerated germination. (2) Application of carbon always accelerated germination; but the pattern of response varied in different treatment combinations. (3) Alkali always decreased the rate of germination in sealed systems in the absence of carbon. (4) Alkali decreased the rate of germination in sealed systems in the presence of carbon in one out of four trials.

### (d)  Varietal Response

The extent to which the above findings apply throughout the species is indicated by the data of Table 8 for a number of standard strains, together with introductions from the Mediterranean region. The trial was conducted approximately 4 months after harvest, by which time a natural spread of dormancy already existed. The strains are listed in Table 8 in decreasing order of dormancy as judged by the final control germination.

In all cases treatment with carbon dioxide brought about prompt and practically complete germination. Treatment with activated carbon or low temperature also increased both the speed and amount of germination; but not so markedly as did carbon dioxide. Of these latter two treatments there appeared a tendency for carbon to be the more effective on the more dormant samples, and cold on the less dormant.

### IV.  DISCUSSION

It seems warranted to conclude that the germination responses described above, to both carbon dioxide and activated carbon, occur throughout the species.

The suggestion that activated carbons produce their effects by production of carbon dioxide can scarcely be regarded as proven, and it is clear that direct proof would be difficult to obtain. However, the case for this hypothesis is relatively strengthened by the following considerations which militate against the possibility of inhibitor adsorption: (1) There is little direct evidence for the existence of an inhibitor. Such an inhibitor would almost certainly be water soluble; but in this work leaching with water for periods up to 6 days did not produce clear-cut effects on germination which could be referred unambiguously to the leaching itself. It is most probable that any possible inhibitor is not located in the testa (where such inhibitors are usually, though not invariably, to be found), for it has been observed that, on occasions when the usually efficacious dormancy-breaking treatment of testa removal failed, the embryos could be promptly germinated by covering with activated carbon. (2) The rapidity of action of the carbons renders inhibitor adsorption less likely, since it must now be assumed that any inhibitor would have to diffuse from the embryo through the testa. (3) Considerable variation in the response to carbon was observed in the experiments involving sealing of vessels and placement of alkali. Whilst these inconsistencies cannot be fully interpreted, they

probably reflect such complexities in the system as the formation and breakdown of carbon complexes, self sorption by the carbon of carbon dioxide, and diffusion-limited absorption of carbon dioxide by the alkali. Had only the adsorption of an inhibitor been involved, a uniform response to carbon would be expected, and this was not found.

This appears to be the first occasion for which such an explanation has been suggested for the action of activated carbons, an interpretation which focuses attention on the action of carbon dioxide as the primary phenomenon. Carbon dioxide is usually held to induce secondary dormancy (Thornton 1953), and stimulation of germination by it has only rarely been recorded (Anderson 1933; Thornton 1935, 1936). However, in these cases, much higher concentrations of carbon dioxide

TABLE 9

EFFECT OF AGGREGATION OF SEED ON GERMINATION

Values are per cent. germinations at day 4 of test. Mt. Barker strain

| No. Seeds per Pile | Seeds Only | Seeds + "Primer" |
|---|---|---|
| 1 | 1·4 | 14·4 |
| 5 | 50·0 | 74·7 |
| 15 | 96·0* | 96·2* |

*No significant difference. All other possible comparisons significant at $P < 0.001$.

were involved—for both *Xanthium* and lettuce the lowest effective concentration reported by Thornton was 5 per cent., and the effectiveness increased under certain conditions as the concentration rose to 80 per cent. This contrasts sharply with the very low concentration effective on subterranean clover, and it seems that quite distinct phenomena are involved. Discussion on possible mechanisms involved in the action of carbon dioxide will be deferred to a later paper where data on temperature relationships will be presented.

This extreme sensitivity of subterranean clover seeds to traces of carbon dioxide suggests that it may be made the basis of a dormancy-breaking treatment valuable in routine seed testing. In any event, it is clear that considerable variation in germination must result from hitherto unsuspected variables, such as the use of open germination trays compared with petri dishes, the number of seeds in relation to the capacity of germination container, the frequency of inspection, the intervals at which germinated seeds are removed, and the precise composition of the atmosphere in the incubators. The data of Table 9 are presented as an extreme case. Individual seeds or aggregations of five or 15 seeds were placed in small petri dishes of approximate capacity 7 ml, one seed or aggregate per dish. In a similar set the germination was "primed" by placing a seed previously germinated to have a radicle approximately 2 mm long beside the single seed, or at the centre of the aggregation.

The treatments were extensively replicated. The quite dramatic variation in germination recorded fully supports the above conclusion.

Lime-pelleted subterranean clover seeds germinate more rapidly and produce larger seedlings than non-treated seeds (Myers, unpublished data), and this appears to be another example of differences in germination behaviour interpretable on the basis of data presented in Section III.

One further implication merits mention. Some mechanism to ensure delayed and irregular germination appears to be common in the seeds of wild annual plants. This character has largely been lost in species of agronomic value in the course of their domestication, as a result of either unconscious or deliberate selection. Delayed germination in subterranean clover may be desirable or undesirable according to the location in which it is grown, and the management adopted, and attention may be directed to this character in breeding programmes.

The data collected by Russell (1950) show clearly that the soil atmosphere, especially under pasture, contains carbon dioxide to at least the minimal concentration required to initiate germination in imbibed, dormant subterranean clover seeds, and usually beyond this into the optimal range. It seems, therefore, that, under field conditions, once subterranean clover seeds soften, they will imbibe water ·at the next opportunity, and then germinate promptly. (Under certain conditions the most highly dormant varieties only may not show this pattern, nor need it be seen if soil temperatures are too high—provisos to be amplified in a later paper.) Preliminary tests have shown that imbibed seeds, planted in potting soil, germinate and establish fully at times when laboratory germination trials indicate considerable residual dormancy. If this conclusion is correct, it would appear more profitable to direct attention to the character of hard-seededness, rather than dormancy, in any attempts to modify varietal characteristics of delayed germination.

## V. Acknowledgments

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# THE INHERITANCE AND ECOLOGICAL SIGNIFICANCE OF SEED DORMANCY IN SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM* L.)

By F. H. W. Morley*

## Summary

Seed dormancy in subterranean clover (*Trifolium subterraneum* L.) was shown to be dependent, at least in part, on the genotype of the embryo, as distinct from that of the testa. Analyses of data from $F_2$'s, and from a diallel, demonstrated that degree of dormancy was highly heritable. The average results of seeds from $F_1$ plants were similar to those from the mid-parent. But results from four sets of $F_2$ plants indicated a tendency for dominance of high dormancy.

Dormancy was expressed in soil, but much less than in laboratory tests. Results from the soil approximated those obtained in the laboratory, at similar temperatures, in an atmosphere containing 6–8 per cent. carbon dioxide. Seeds which were dormant in soil remained viable and dormant at 25°C in petri dishes, despite three cycles of wetting and drying during which they were held moist at 25°C for a total of 14 days or more. These results indicate that loss of seed following summer rains may be prevented, to some extent, in some strains, by seed dormancy.

A survey of germination percentages at 20–22°C of a number of strains from the Mediterranean region showed that, with several exceptions, dormancy tended to be greater in strains from cool climates than in those from warmer climates. The agronomic significance of seed dormancy is discussed.

## I. Introduction

Great importance has been attributed by Went (see Juhren, Went, and Phillips 1956), Evenari (1949), Koller (1955), and others to the regulation of germination in adaptation of plants to particular environments.

Germination of seeds of many species has been shown to be wholly or partly dependent on temperature. Seeds of some species germinate well at high temperatures but poorly at low temperatures. In other species the reverse is true. In general, seeds of species which normally germinate in the autumn and grow through the winter have low temperature optima. Germination of seeds of species which normally commence growth in spring is usually inhibited by low temperatures.

Toole and Hollowell (1939) and Loftus Hills (1944a) demonstrated that high temperatures inhibit germination of subterranean clover. Ballard (1958) examined the effect of temperature and other factors in detail, and demonstrated that at temperatures above certain limits, depending *inter alia* on strain and age of seed, germination of some seeds was inhibited. In this paper the term "dormancy" is used to refer to inhibition of germination by high temperatures.

This paper presents results of experiments designed to investigate the inheritance and ecological significance of seed dormancy in subterranean clover (*Trifolium*

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*subterraneum* L.). The problem of inheritance is considered by examination of the location of genetic differences in testa or embryo and by a biometrical analysis of a diallel cross. The ecological significance is examined by testing whether dormancy is expressed in soil, whether seed loss may be prevented by dormancy, and whether the distribution of dormancy is related to habitat. Finally, the mechanism of determination of differences between strains and the agronomic significance of seed dormancy are discussed.

## II. METHODS AND MATERIALS

All tests were made on replicates of 25 seeds each. Thirty seeds were placed on moist "Wettex" pads in petri dishes. Three or four hours later those seeds which had not swollen were scratched lightly with a needle. Seeds which had not imbibed or which became mouldy were replaced from the spare five included for this purpose.

Dishes were inspected each day and all seeds with protruding radicles were classed as "germinated" and removed. After 5–7 days, germination at the temperature of the test was virtually asymptotic. The percentage germination was recorded and the arc sine transformation of that figure used in analysis of variance.

All seeds of each replicate were obtained from a single plant grown at Dickson Experiment Station, A.C.T., during 1955 or 1956. Plants were spaced 7 links apart, in rows 7 links apart. Mature seeds were harvested in December and January and tests were made in February–April.

## III. RESULTS AND DISCUSSION

### (a) *Inheritance of Seed Dormancy*

(i) *The Site of Determination.*—In a sample of seeds a certain proportion will germinate under a given set of conditions. Those which germinate differ from those which do not because of some factor or factors in the testa, the embryo, or both. These factors may be genetically or environmentally determined. If genetic differences in embryos are responsible, we might expect progeny of the germinators and non-germinators to differ to an extent depending on the selection differential and the relative sizes of genetic and environmental effects. If we take embryos which differ in genotype, but which are contained in testas which do not differ genetically, we can test the null hypothesis that germination is independent of the genotype of the embryo. These conditions are fulfilled by $F_2$ seeds from $F_1$ plants, since the testa is of maternal origin.

Twenty-five well-developed seeds from each of four $F_1$ plants were placed in dishes, with moisture, at room temperature. Seeds which germinated within 5 days formed an "active" group. Those which did not are described as the "dormant" group. Plants from these seeds were grown at Dickson Experiment Station, and germination tests were performed at 20°C on seeds from these plants and from eight plants of each parent strain. Two replicates were used, one dish of 25 seeds from each plant forming one replicate. The results of these tests are presented in Table 1, and analyses of variance in Table 2.

The differences are individually not significant, but are of the same sign, and in the direction expected if germination is not independent of the genotype of the

embryo. If germination were independent of the genotype of the embryo the probability of obtaining results such as those of these four experiments may be found by a modification of a procedure discussed by Cochran (1954) and described by Yates (1955). The probability associated with each $F$ value (through $\sqrt{F} = t$) was transformed to the equivalent normal deviate with the appropriate *sign*. The sum of $k$ normal deviates is distributed normally with standard deviation $\sqrt{k}$. Therefore this sum divided by $\sqrt{k}$ is normally distributed with unit variance. The probability of obtaining a deviate equal to this or larger may be found from tables of the integrated normal distribution (using a two-tailed probability). Using this procedure

TABLE 1

PERCENTAGE GERMINATION OF SEED FROM PARENTS AND $F_2$ PLANTS

| Genotype | Active | | Dormant | | Total | | Mid-parent |
|---|---|---|---|---|---|---|---|
| | No. of Plants | Germination (%) | No. of Plants | Germination (%) | No. of Plants | Germination (%) | |
| Tallarook × Burnerang $F_2$ | 10 | 17 | 13 | 14 | 23 | 15 | 44 |
| Tallarook × Northam First Early $F_2$ | 6 | 48 | 16 | 27 | 22 | 33 | 48 |
| Bacchus Marsh × Burnerang $F_2$ | 6 | 18 | 11 | 6 | 17 | 10 | 31 |
| Bacchus Marsh × Northam First Early $F_2$ | 5 | 25 | 20 | 15 | 25 | 17 | 34 |
| Tallarook | | | | | 8 | 88 | |
| Burnerang | | | | | 8 | 1 | |
| Bacchus Marsh | | | | | 8 | 61 | |
| Northam First Early | | | | | 8 | 8 | |

the probability of chance occurrence of such a result was found to be 0·02. The hypothesis that germination is independent of genotype of embryo was therefore rejected. This should not be taken to indicate that differences between strains are determined wholly by differences in activity of embryos of the seed. For example, the amount of an inhibitor produced by the parent plant and stored in the seed might vary between strains.

(ii) *A Diallel Analysis.*—The variance among individuals in a population may be divided into heritable and environmental portions. In turn, the heritable portion may be divided into various fractions by various techniques. Griffing (1956a) showed that diallel analyses may be used to distinguish additive and non-additive components of heritable variation. The former, that fraction which, in a random breeding population, is estimated from the parent–offspring regression, may be estimated from $2\sigma^2$ g.c.a. (general combining ability) as defined by Griffing. The latter, estimated as $\sigma^2$ s.c.a. (specific combining ability) by Griffing, includes dominance deviations and interactions between loci.

TABLE 2

ANALYSIS OF VARIANCE OF ARC SINE GERMINATION PERCENTAGE IN $F_2$'S AND PARENT STRAINS

| Source of Variation | $F_2$'s | | | | | | | | | | Parent Strains | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tallarook × Burnerang | | Tallarook × Northam First Early | | Bacchus Marsh × Burnerang | | Bacchus Marsh × Northam First Early | | Pooled† | | Source of Variation | | |
| | D.F. | M.S. | D.F. | M.S. | D.F. | M.S. | D.F. | M.S. | D.F. | M.S. | | D.F. | M.S. |
| Replicate | 1 | 226 | 1 | 1 | 1 | 0 | 1 | 622 | 4 | 119 | Replicates | 1 | 25 |
| Active v. dormant | 1 | 119 | 1 | 2111 | 1 | 443 | 1 | 251 | 4 | 824 | Strains | 3 | 17368** |
| Replicate × treatment | 1 | 82 | 1 | 14 | 1 | 16 | 1 | 4 | 4 | 29 | Replicates × strains | 3 | 396 |
| Plants in $F_2$ | 21 | 244 | 20 | 623** | 15 | 265 | 23 | 575** | 79 | 440** | Plants in strains | 28 | 201* |
| Residual | 21 | 150 | 20 | 147 | 15 | 120 | 23 | 98 | 79 | 129 | Residual | 28 | 102 |

*$P<0.05$.   **$P<0.01$.   †Pooled sums of squares and degrees of freedom from each $F_2$.

In a study such as this, in which applications in plant breeding are relevant, the relative values of variances attributable to g.c.a., s.c.a., and environmental factors are of value as an indication of the degree of resemblance between parent and offspring; that is, the value of the phenotype as an index of genotype. From the viewpoint of selection in segregating material, if g.c.a. variance is large relative to the other fractions, results of crosses may be predicted with reasonable precision, decisions may be based on the performance of single plants, and progress should be rapid.

If s.c.a. variance is relatively large the performance of crosses may not be predicted with accuracy and, because the parent–offspring regression will be relatively small, selection of single plants, or plants from a specific cross, may not be very

TABLE 3

PERCENTAGE GERMINATION IN DIALLEL

| Female Parent | Male Parent | | | | | | |
|---|---|---|---|---|---|---|---|
| | Tallarook | Mt. Barker | Burnerang | Cranmore | Bacchus Marsh | Northam First Early | Dwalganup |
| Tallarook | 88 | 74 | 31 | 61 | 42 | 57 | 89 |
| Mt. Barker | 62 | 27 | 42 | 20 | 45 | 17 | 54 |
| Burnerang | — | — | 12 | 18 | 12 | 10 | 49 |
| Cranmore | 67 | 28 | — | 55 | 81 | 8 | 61 |
| Bacchus Marsh | — | — | — | — | 32 | 35 | 93 |
| Northam First Early | 63 | 26 | — | 32 | — | 15 | 37 |
| Dwalganup | — | — | — | — | — | — | 88 |

effective. If the environmental variance is large, increased replication may be necessary, and selection must be based on line rather than single plant performance. This would place limitations on the selection differential.

The analysis is also of interest as an indication of gene action. If s.c.a. variance is small, relative to g.c.a. variance, dominance and gene interactions must be small relative to additive effects of genes. If s.c.a. variance is relatively large one or both these non-additive effects may be large.

Seven strains (Tallarook, Mt. Barker, Burnerang, Cranmore, Bacchus Marsh, Northam First Early, and Dwalganup) were crossed in all combinations. Each parent and cross was tested in four replications, two from each of two $F_1$ plants where available, otherwise the four replicates were taken from one plant. The only reciprocal crosses available were among Tallarook, Mt. Barker, Cranmore, and Northam First Early and these were tested. The means obtained from all tests are given in Table 3.

A test was made for differences between reciprocal crosses by analysis of the $4 \times 4$ diallel among Tallarook, Mt. Barker, Cranmore, and Northam First Early, using a modification of a technique described by Griffing (1956b). The analysis, given in Table 4, indicates that no differences were present, a result which would be expected if the genotype of the embryo determined dormancy, or if differences, if any, between reciprocal $F_1$'s were not imposed upon their seeds.

Estimation of g.c.a and s.c.a. by Griffing's methods is not entirely valid in this material for, as shown in the previous section, the means from seeds of $F_1$ plants are, to some extent, means of $F_2$'s and not strictly $F_1$ values. Nevertheless, since Griffing has shown the nature of bias introduced (see Davern, Peak, and Morley 1957),

TABLE 4

ANALYSIS OF DIFFERENCES IN GERMINATION BETWEEN RECIPROCAL CROSSES

Data transformed to arc sine

| Source of Variation | D.F. | M.S. | E [M.S.]* |
|---|---|---|---|
| Replicates | 1 | 1 | —— |
| General combining ability | 3 | 3350 | $E+2 \cdot 1\ P+2S+4G$ |
| Specific combining ability | 2 | 1 | $E+2 \cdot 1\ P+2S$ |
| Reciprocal effects | 6 | 265 | $E+2 \cdot 1\ P+k_1R$ |
| Plants | 10 | 266 | $E+2 \cdot 1\ P$ |
| Residual | 25 | 92 | $E$ |

*In this and the following table the letters under E [M.S.] signify the variance components appropriate to the source of variation in column 1.

the $7 \times 7$ diallel was analysed by Griffing's (1956b) model II, method 4, with results given in Table 5. This model was used as I wished to apply results to a population of crosses among strains, of which these seven might be considered a random sample.

In this diallel analysis both g.c.a. and s.c.a. variances were significant, and large relative to environmental variations. Using assumptions described by Griffing, specific effects were estimated and could largely be attributed to crosses with Bacchus Marsh, there being a large negative effect in the cross with Tallarook and large positive effects in crosses with Cranmore and Dwalganup. Bacchus Marsh was removed from the diallel and the new $6 \times 6$ diallel analysed with results presented in Table 6.

This modification of the analysis resulted in removal of the non-additive component of variation, which indicates that a gene (or genes) in this strain interacts with genes at the same or different loci in some other strains. Otherwise, regarding the six strains used as a random sample of inbred lines formed from a random breeding population, one may state that genetic variation is largely additive. The validity of this statement is scarcely affected by whether the values in the diallel are $F_1$'s or means of $F_2$'s.

There is an inconsistency between this result and those from the $F_2$'s, described in the previous section. The $F_1$ of Tallarook $\times$ Northam First Early has approximately

the same germination percentage as the mid-parent value ($F_1=60$, mid-parent$=52$). The $F_2$ mean is 33 and mid-parent 48, the difference being highly significant. The means for all four $F_2$'s are highly significantly less than mid-parent values, suggesting dominance of dormancy, which is not consistent with results from the diallel, in which $\overline{F}_1=46$ and $\overline{P}=45$. A method of reconciling these results is not obvious.

TABLE 5

ESTIMATION OF VARIANCE COMPONENTS FROM THE DIALLEL CROSS

Data transformed to arc sine

| Source of Variation | D.F. | M.S. | E [M.S.] | Estimate of Component |
|---|---|---|---|---|
| Replicates | 1 | 40 | — | — |
| General combining ability | 6 | 2522 | E+2·14 P+S+5G | 390 |
| Specific combining ability | 14 | 570 | E+2·14 P+S | 361 |
| Plants | 18 | 209 | E+2·14 P | 55 |
| Residual | 44 | 91 | E | 91 |

The statistics of Tables 2 and 6 are consistent in demonstrating that genetic variation is substantially larger than variation from environmental sources. That is, selection for or against dormancy should be simple and effective. Three levels of selection may be practised—between seeds, between plants, and between lines—and different sources of environmental variation will have different effects on these.

TABLE 6

ESTIMATION OF COMPONENTS IN $6 \times 6$ DIALLEL (AFTER REMOVAL OF BACCHUS MARSH)

| Source of Variation | D.F. | M.S. | Estimate of Component |
|---|---|---|---|
| Replicates | 1 | 20 | — |
| General combining ability | 5 | 2267 | 507 |
| Specific combining ability | 9 | 239 | 4 |
| Plants | 11 | 235 | 61 |
| Residual | 33 | 98 | 98 |

Selection between $F_2$ seeds seems, from Table 1, relatively ineffective, presumably because of environmental effects peculiar to individual seeds. There are two sources of error affecting values for individual plants. The first of these, the "residual" variance (E) of Tables 2 and 6, consists of the sampling variance associated with number of seeds per dish and, in addition, effects due to differences between samples (associated with a particular dish). The sampling variance should be $820·7/n=33$

(Fisher and Yates 1949), if $n$ is the number of seeds per sample. The dish component is therefore approximately 70. The second source of error is the environmental effects peculiar to plants (P). This component was found to be approximately 50–60 (Tables 2 and 6).

The heritability of a germination percentage would be approximately

$$G/(G+55/p+70/pd+820 \cdot 7/npd),$$

where $G$ is the appropriate genetic variance, $p$ is the number of plants per unit, $d$ the number of dishes per plant, and $n$ the number of seeds per dish. There is therefore little to be gained from increasing the number of seeds per dish beyond 20. The main increases in accuracy are likely to come from increasing the number of plants tested per line and, to a less extent, the number of dishes per plant. Single plant records are inevitably of limited accuracy. Nevertheless since, in the $F_2$ studied (Table 2), $G$ averages about 120, substantial progress should be possible from selection within such $F_2$'s.

The fact that dormancy is affected by environmental factors acting on the maternal plant suggests that a substance produced by the plant, and stored in the developing seed, determines dormancy. In the previous section evidence was presented that the genotype of the embryo was involved. Possibly the substance determining dormancy is produced by the embryo from substrate provided by the parent plant. An alternate theory will be presented in the general discussion of this paper.

### (b) The Ecological Significance of Dormancy

(i) *Survival of Seeds in Soil.*—If dormancy is a mechanism for seed conservation it is, first, necessary that it should be effective in soil and, second, that seeds should survive one or more cycles of imbibing and drying without substantial loss of viability.

Tests were made with five strains to determine whether dormancy was expressed in the soil. Four lots of 100 seeds each, of each strain, were folded in sheets of coarse blotting-paper so that they were distributed along about 50 cm of blotting-paper. This was secured by paper fasteners and buried in a sandy loam, 2–3 cm deep, near the Plant Industry Laboratories at Canberra. The surface of the soil was covered with a thin layer of straw. Soil thermometers were placed in several positions in and around the "plot" and a soil thermograph, with the thermometer wrapped in blotting-paper and buried in the plot, was used to obtain a continuous record of soil temperatures. The whole plot was watered liberally and kept moist. The soil temperature varied from 16 to 28°C with a mean close to 20°C. (This could not be determined accurately because of a fault in the clock mechanism of the thermograph, resulting in loss of the continuous record of one day.)

Tests on other samples from the same source were made in the laboratory at 25°C and at room temperature (average about 20°C), and at these temperatures in an atmosphere containing 6–8 per cent. $CO_2$. The $CO_2$ treatments were included because Ballard (1958) found that at such concentrations, and even at 0·3 per cent., this gas strongly stimulated germination of dormant seeds of subterranean clover, and because the soil atmosphere is likely to contain a relatively high concentration of

$CO_2$. On the 4th, 5th, 6th, and 7th days one lot of each strain was dug up and the number of germinated, hard, and dormant seeds counted. The results of these tests are given in Table 7.

Germination in all strains was much higher in the soil than in the laboratory at either 25°C or room temperature. The results in soil were similar to those obtained at an equivalent temperature in an atmosphere enriched with $CO_2$. Although this does not prove that the stimulant in the soil was $CO_2$, the results are highly suggestive. $CO_2$ was highly effective with both Wenigup and Tallarook, but was practically ineffective at 25°C with Northam First Early, Burnerang, and Mt. Barker. This result also demonstrates that $CO_2$, although a strong germination stimulant, is not strong enough to overcome dormancy at high temperatures.

TABLE 7

GERMINATION PERCENTAGES OF IMBIBED SEEDS AS AFFECTED BY SOIL, CARBON DIOXIDE, AND TEMPERATURE

Readings taken at 7 days

| Treatment | Strain | | | | |
|---|---|---|---|---|---|
| | Wenigup | Tallarook | Mt. Barker | Northam First Early | Burnerang |
| Field | 100 | 100 | 97 | 36 | 34 |
| (Percentage hard seed) | (2) | (10) | (1) | (2) | (50) |
| 25°C+CO₂* | 100 | 93 | 11 | 0 | 4 |
| 20°C+CO₂† | No test | 100 | 99 | 62 | 45 |
| 25°C | 57 | 11 | 2 | 4 | 2 |
| Room temp. (av. c. 20° C)‡ | 92 | 63 | 5 | 1 | 7 |

*Reading on day 5 when test terminated.
†Continuation of 25°C+CO₂; reading on day 11.
‡Samples transferred to controlled temperature (19–20°C) on day 5.

The ability of dormant seeds to retain viability despite repeated cycles of moistening and drying was then tested. 263 seeds of Northam First Early and Burnerang which had remained dormant (though imbibed) in the soil were dried and were divided into three groups. One received no further treatment. The second and third were held moist for 4 days at 25°C, during which four of 179 seeds germinated. The dormant seeds were dried and then the third group was moistened and held moist for a further 6 days at 25°C, during which none germinated.

Viability of all groups was then tested by stimulation of germination by low temperatures and exposure to 6–8 per cent. $CO_2$ in air. All but three seeds germinated, one of these being mouldy. The other two germinated when testas were removed.

These results demonstrated the ability of dormant seeds to retain viability and dormancy despite repeated wetting and drying. If dormancy were determined by an inhibitor present in the seed, the inhibitor is apparently relatively insoluble or non-diffusible (or both).

These experiments, together with those of Ballard (1958), demonstrate that germination is stimulated by low temperatures, the presence of $CO_2$, and some factor (which may be $CO_2$) which is present in active concentrations in soil. I am not aware of any observation, in field or laboratory, which is inconsistent with these results. I do not suggest that other factors, such as degree of moistening, are unimportant. Neither $CO_2$ nor the soil factor can wholly overcome inhibition of germination by high temperatures.

(ii) *Distribution of Dormancy.*—Variations among strains in high temperature inhibition of germination presumably have arisen as adaptations to ensure survival of strains in their natural habitats. The mechanism is probably valuable as it prevents germination at times when seedlings would be unlikely to survive—in Mediterranean climates this time would be during summer. Germination would be prevented during summer months, even if moisture were available, by high temperatures. As temperatures fall during autumn, in Mediterranean climates the probability of available moisture becomes higher and the survival of seedlings more assured. One would expect that dormancy would inhibit germination at temperatures above those normally encountered at the time of opening rains. The temperature at which germination is inhibited would therefore tend to be lower for strains from cooler and drier habitats.

This hypothesis was tested by germination tests on several strains obtained from two collecting expeditions in the Mediterranean area. Tests were made during February to April 1956 and 1957 using seeds from plants grown at Canberra the previous season, and harvested during December–January. At least 100 seeds, and usually 200, were tested in at least four replicates at 20–22°C. The percentage germination at this temperature is assumed to reflect the ranking which would have been obtained if the temperatures at which a certain percentage germination occurred had been measured. Although I have evidence on this point, it will not be presented as Ballard (1958) has more extensive evidence for presentation. The percentage germination is also assumed to indicate the relative expression of dormancy (the actual level may depend on soil) in the native habitat. From meteorological data, obtained by Mr. R. J. Williams, the habitats were classed as warm or cool, dry or moist. This classification is necessarily only approximate since recording stations were frequently several miles from collecting localities. Further, microclimatic and other factors affecting temperature and availability of moisture could not be considered.

A summary of the results of these tests is presented in Table 8. Analyses of variance on these percentages (transformed to the arc sines), ignoring the moist-dry classification, disclosed:

(1) Differences between collections within climate were not significant for the warm class but were for the cool class. If the collection from Greece was eliminated from the analysis, differences between collections were not significant within the cool class.

(2) The warm–cool difference was significant at $P < 0.05$. If the collection from Greece was eliminated the difference was significant at $P < 0.001$.

The results therefore support the hypothesis that higher levels of dormancy are associated with cooler climates. They do not support the hypothesis that higher levels of dormancy are associated with drier climates. A possible explanation of this result is that summer rains are practically non-existent in the more arid parts of the Mediterranean area, so that dormancy may not be necessary in such regions. The relationship of dormancy to moisture may be non-linear.

The discrepancy between results from Greece and others in the cool class is of particular interest. The strains were all collected from a hilly to mountainous region in northwest Greece. This region has a high rainfall (over 40 in.) with more

TABLE 8

GERMINATION PERCENTAGES OF STRAINS FROM DIFFERENT LOCALITIES

| Region | Climate of Locations | | | |
|--------|----------|-----------|----------|------------|
| | Warm Dry | Warm Moist | Cool Dry | Cool Moist |
| Morocco | 14, 70, 70, 72, 83, 86 | | 18 | 10, 26, 26, 32, 37 |
| Portugal | 39, 68, 70 | 12, 33, 46, 60 | | 12, 12, 32, 56 |
| Algeria | 56, 88 | 18, 79, 89 | | 35, 48 |
| Greece | | | | 8, 20, 34, 40, 48, 50, 50, 74, 76, 76, 79, 80 |
| Cyprus | | | 8, 8 | |
| Turkey | | | 8, 12, 21, 23 | |
| Canary Is. | 46 | | | |
| Israel | 50, 76 | | | |
| Mean | 63 | 48 | 14 | 42 |

summer distribution than collections from other localities. In such circumstances the requirement for dormancy would be less compelling, especially in locations with especially favourable moisture relationships through soil, topography, or drainage, for survival of seedlings would be less hazardous. Further, as shown by Hilder (personal communication) in the cool, moist New England region of New South Wales, rapid germination and growth following midsummer rains is desirable to ensure that plants are well established before the winter, so that dormancy could be disadvantageous. This may be the situation in the cool, moist regions of Greece.

Differences found within the species in this study are comparable to those reported by Juhren, Went, and Phillips (1956) between species from a transect of part of the Californian desert.

## IV. General Discussion

This and other studies on dormancy in subterranean clover have shown that it is influenced by the following factors:

(i) Environmental factors acting on the parent plant.

(ii) Age of seed—dormancy declines with age (Loftus Hills 1942).

(iii) Conditions of storage—decline in dormancy is accelerated by high temperatures (Loftus Hills 1942).

(iv) Temperature at the time of germination test.

(v) Presence of some factor in soil which partly overcomes dormancy. It has an effect similar to that of $CO_2$.

(vi) Removal of the seed coat.

(vii) A substance or substances produced by the embryo varying in activity or amount according to the genotype of the embryo.

The theory which seems most in accord with these facts is that an inhibitor is present in the seed, having been deposited there by the parent plant. This inhibitor is somewhat unstable or is metabolized or inactivated during storage, the process involved having a positive temperature coefficient. Removal of the testa either removes the inhibitor, or allows it to diffuse away from the embryo. Dormancy is counteracted by a substance produced by the embryo which overcomes in some way the action of the inhibitor. This process has a negative temperature coefficient, so that it is unlikely to be a simple chemical action. Carbon dioxide, and other promoters of germination, may either inactivate the inhibitor or promote formation of the anti-inhibitor by the embryo, or both.

The difference between dormant and non-dormant strains may largely depend on a difference in balance between the production and distribution of inhibitor by the parent plant and the production of the promoting substance by the embryo. Since dominance is incomplete, even if only two processes were involved, a number of different degrees of dormancy would be possible. Some of these might be indistinguishable from others, since the same degree of dormancy could be achieved through different pathways.

It is more likely that more than two processes are involved, and modifications may be readily visualized. For example, the ratio of embryo to testa varies widely between strains (Morley, unpublished data) and this could affect the amount of inhibitor which could be stored and the rate of its inactivation by substances produced by the embryo. It is not surprising that the survey of strains showed a reasonably continuous distribution of dormancy.

The evidence is at present insufficient to discriminate between this and other propositions. The absence of an inhibitor from the embryo, and its presence in the endosperm or testa, as found by Lasheen and Blackhurst (1956) in the blackberry, is not conclusive evidence that an inhibitor is not produced by the embryo. Further as these authors point out, the presence of inhibitors in the embryo may be masked by a large amount of promoting substance. Dormancy may be the resultant of disappearance of inhibitors and formation of promoters, but the part played by

the embryo in this process is obscure. We can only state that the embryo does play some part in subterranean clover.

Conservation of subterranean clover seed may be achieved by two mechanisms, dormancy and hard-seededness. The first is relatively transitory since it can be of importance only in the year following seed setting. Long-term conservation is more dependent on hard-seededness, and is probably of far greater adaptive significance since, especially in annuals, prolonged viability of seed is important. This may account for the results of Loftus Hills (1944*b*) who found that varietal differences were much less evident for hard-seededness than for dormancy.

The agronomic significance of dormancy has been discussed by Loftus Hills (1942, 1944*c*) who considered that high levels might be desirable in certain special cases. He drew attention to the compromise between rapid germination on the one hand and survival of seeds during summer on the other.

In the subterranean clover belt of south-eastern Australia the summer rainfall is frequently almost as high as the winter rainfall, but much less reliable. In the drier areas of this region some level of embryo dormancy is probably very desirable. On the other hand, in cooler and moister regions, especially those with a high and reliable summer rainfall, embryo dormancy may well be detrimental to productivity, although not necessarily to survival. In regions with completely dry summers dormancy may be unimportant for survival. The survival in Western Australia of Dwalganup, which has very little dormancy, is therefore not surprising. It is, however, doubtful if this strain would survive as well in the drier parts of the south-east where rainfall has a far more erratic distribution.

Whilst the level of expression of dormancy in the native habitat seems to be largely associated with temperature, the fact that most of the relevant habitats were characteristically Mediterranean in rainfall distribution must be emphasized. In Australia, dormancy is likely to be important wherever unreliable summer rains are likely to cause seed wastage. Conversely, it may be undesirable where summer rains are reliable and unnecessary where summer rains are absent.

## V. Acknowledgments

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# STUDIES ON PHYTOALEXINS

## I. THE FORMATION AND THE IMMUNOLOGICAL SIGNIFICANCE OF PHYTOALEXIN PRODUCED BY PHASEOLUS VULGARIS IN RESPONSE TO INFECTIONS WITH SCLEROTINIA FRUCTICOLA AND PHYTOPHTHORA INFESTANS

### By K. O. Müller*

### Summary

In using the inner epidermis of *Phaseolus vulgaris* pods as host tissue and *Sclerotinia fructicola* and *Phytophthora infestans* as pathogens which interact in a hypersensitive fashion, a principle (or principles?) was separated from the former which exerts a strongly inhibitory effect on the growth of the latter. It is shown that this principle (phytoalexin):

(i) Is the result of an interaction between host and pathogen and is absent from non-infected host tissue at concentrations which could exert an inhibitory effect on the pathogen.

(ii) Is formed at a rate and at concentrations which are sufficient to prevent further growth of the pathogen in the diseased tissue.

(iii) Is not specific, and

(iv) Possesses properties from which its nature as an individual chemical factor(s) becomes obvious.

Concerning the action of the phytoalexin(s) under consideration, results as follows were obtained:

(i) The antibiotic activity is not influenced by chemical factors in the host tissue which may serve as nutrients to the pathogen.

(ii) Within a pH range of 4·0–7·5, the hydrogen ion concentration exerts neither an antagonistic nor a synergistic effect on the activity of the inhibitory principle.

(iii) There is strong adsorption of the active principle to non-parasitized cells.

(iv) The output per unit volume of parasitized tissue is dependent on the age of the host tissue.

Six other host–pathogen combinations, which interact in a hypersensitive fashion, were shown to produce, post-infectionally, inhibitory principles at concentrations sufficient to stop the pathogen's growth.

In the light of these results, the mechanism is discussed which underlies the local lesion reaction. It is stressed that—at least in the cases under discussion—"resistance" is preconditioned by the ability of the host tissue to encounter the metabolic activities of the pathogen with the formation and the accumulation round the infection sites of an antibiotic principle which has been termed "phytoalexin".

## I. Introduction

The problem of disease resistance in plants has exercised the minds of phyto-pathologists for more than half a century. More than 50 years ago Ward (1902), investigating the behaviour of *Puccinia dispersa* in uncongenial hosts, discovered

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that shortly after penetration the pathogen ceased to spread; simultaneously, the host tissue underwent necrotic changes and a "local lesion" resulted at the point of infection. In considering the fundamental cause of the checking of the pathogen in necrotic tissue Ward mooted the possibility that preformed toxins present in the host might be the actual cause of the arrest of the pathogen. But he failed to demonstrate the presence of such factors in extracts from plants which responded in this way and finally concluded that the antagonism must be due to something much more subtle "than a mere soluble poison oozing from the cells". He suggested that "the onslaught of the pathogen on the resistant host cells is too vigorous", and therefore the infected cells die too rapidly, and since dead cells are unsuitable as a medium for further growth of the mycelium, the parasite dies. Nevertheless, he maintained the view that enzymes or toxins might be involved in the local lesion reaction.

Subsequent authors (e.g. Gibson 1904; Marryat 1907; Stakman 1914; and Allen 1923, 1927) also considered the possibility that toxins and antitoxins were involved in the local lesion reaction, but due to lack of experimental evidence the toxin concept gradually lost ground, and became largely displaced by the nutritional hypothesis, which was fostered by quite a number of workers (e.g. Leach 1919; Wellensiek 1927). In this concept the non-establishment of the pathogen in resistant hosts was explained on the grounds that specific nutrients required by the parasite were absent from these hosts and thus the fungus died from starvation; subsequently necrosis of the host cells took place due to substances diffusing from the dead parasite. A variation of this concept suggested that diffusion substances from the parasite first killed the host cells and thereafter the parasite died of starvation.

Because of lack of experimental evidence in support of the nutritional hypothesis, however, the idea that there might be substances present or formed in the host tissue which could exert an inhibitory effect on the pathogen, continued to linger in many minds. Angell, Walker, and Link (1930) isolated such a toxic principle, protocatechuic acid, from scales of onion varieties resistant to onion smudge (*Colletotrichum circinans*). This toxin, however, is produced only in the dead outer scales of the bulbs and therefore it cannot be directly connected with the local lesion reaction.

About the middle of the thirties, Vavilov wrote a review in which all aspects of the problem of immunity of plants to infectious diseases were discussed. It includes also the Russian literature on the subject and may therefore serve as a useful guide to the understanding of the situation up to that time.

Müller and his co-workers (1939, 1940, 1949, 1950, 1953) and Meyer (1940) used a new approach. Müller and Börger (1940), working with different strains of *Phytophthora infestans* and tubers of resistant and susceptible potato hybrids, found that a *Phytophthora* strain, interacting with the host in a hypersensitive fashion, initiates a change in the parasitized tissue of such a kind that thereafter even virulent strains are not able to establish themselves in the changed tissue. Various strain-host combinations were tested. They always showed a predominance of the less virulent strain when inoculations were made simultaneously. Other

microorganisms, pathogenic and non-pathogenic to the potato tuber, were also checked in tissues which had reacted with an avirulent *Phytophthora* strain (Müller and Börger 1940). With these results the nutritional concept was ruled out as an explanation of the restriction of the pathogen's growth on hypersensitive hosts. However, the change in the host tissue was a completely local one. If the speed of the hypersensitive reaction was reduced by pretreating the host tissue with narcotics, then the spread of the parasite increased (Behr 1949; Müller and Behr 1949). Furthermore it was shown in genetically analysed breeding lines that the spread of the pathogen in the host tissue decreased with increasing reaction rate (Müller 1953). From these results it was postulated that a principle, which was post-infectionally formed and non-specific, was actually responsible for checking the parasite in hypersensitive tissue. This principle was named "phytoalexin", a designation which refers only to the biological (i.e. the defensive) action of the active principle and does not include any suggestion as to its chemical nature (Müller and Börger 1940).

This concept of plants being able to encounter an infection by the formation of "antibiotic bodies" was efficiently supported by subsequent investigations of Gäumann and Jaag (1945) and Gäumann, Braun, and Bazzigher (1950). These workers showed that orchid (*Orchis militaris*) tuber slices exposed to the metabolites of *Rhizoctonia repens* produce a fungistatic substance which has to be regarded actually as a new formation by the living tissues of the orchid tuber. The recent work of Kuč *et al.* (1955, 1956) and Kuč (1957) strongly supports the idea of the plant being able to respond to infections with the production of "antibody"-like chemical compounds. They showed that when slices of potato tubers were inoculated with *Helminthosporium carbonum* an antibiotic factor developed which not only checked *Helminthosporium* but also other fungi. These authors (1956, 1957) found an increased amount of chlorogenic and caffeic acid present in the inoculated tissue. The concentrations found, however, were insufficient for complete inhibition of the parasite *in vitro*. The authors therefore suggested a synergistic effect, *in vivo*, by other cell constituents which are not themselves fungistatic.

Recent work reported from Japan and Australia may be mentioned in this connection. The Japanese workers (Tomiyama 1955, 1956; Tomiyama *et al.* 1956*a*, 1956*b*; Takakuwa and Tomiyama 1957) investigated cytomorphological and chemical changes in the tissue of potato tubers of various degrees of resistance to *Phytophthora infestans*, after infection with that fungus. Their results are in good accordance with those obtained by Müller and his co-workers and support the idea that post-infectional changes of the host tissue are the actual cause of the pathogen being checked in the resistant tubers. In Australia, Scott, Millerd, and White (1957) reported that they isolated from barley leaves a phenolic compound which inhibits the growth of *Erysiphe graminis*. This factor, however, was found to be present in healthy leaves of suceptible varieties as well. The authors therefore suggested that the difference in the reaction of susceptible and resistant varieties was due to the particular sensitivity of the protoplasm of the latter to some metabolites of the pathogen. As "cell collapse is associated with the release of this compound", the metabolic activities of the pathogen's haustoria are prematurely stopped.

Müller (1956) elaborated his phytoalexin theory on purely experimental lines by demonstrating the occurrence of an antibiotic factor in infected tissues under conditions which exclude any doubt that the inhibitory principle is the result of an interaction between host cell and pathogen and is responsible for arresting the growth of the latter in local lesions. Six preconditions had to ·be fulfilled for this purpose:

(1) No preformed substances inhibitory to the pathogens used must be present.

(2) The pathogen must be able to be grown on ordinary nutrient media.

(3) The interaction between host tissue and pathogen (and so the formation of the antibiotic principle) must take place under conditions which exclude interference by contaminants.

(4) Chemical extraction methods which may affect the active principle must be avoided.

(5) Mechanical injury which may bring about the formation of other inhibitory factors must not be involved.

(6) It must be possible to demonstrate that the antibiotic substance is present *in vivo* at concentrations sufficient to stop the pathogen's growth.

Material which complied with these conditions was found in the epidermal tissue of seed cavities of bean pods (*Phaseolus vulgaris*). This tissue reacts to an infection with *Sclerotinia fructicola*, *Phytophthora infestans*, and other fungi, with local lesions. The present paper describes the experiments concerned in more detail, and results obtained will be discussed in relation to work of previous authors in the field of "reactive resistance" in plants.

## II. Material and Methods

The basic test material was the epidermal tissue lining the seed cavities of *Phaseolus vulgaris* pods. Fruits of *Pisum sativum*, *Vicia faba*, and *Capsicum annuum* were also used.

*Sclerotinia fructicola* and *Phytophthora infestans* (strain B) were used as pathogens. Conidia and zoospore suspensions were applied as inocula. The *Sclerotinia* conidia were taken from pure cultures, from 4 to 10 days old, grown on potato dextrose agar; the *Phytophthora* zoospores were obtained from cultures maintained upon living potato tubers.

In addition to the fungi mentioned above, *Colletotrichum lindemuthianum*, *Botrytis cinerea*, *Uromyces trifolii*, *Rhizoctonia solani*, and *Pythium ultimum* were investigated with regard to their sensitivity to phytoalexin obtained from *Phaseolus* pods which had been inoculated with *Sclerotinia* or *Phytophthora*.

The method used to obtain the toxic interaction products has already been described (Müller 1956). It consists of inoculating the seed cavities with spore suspensions and collecting the latter after a certain period of time. The re-collected liquids which have been purified from ungerminated spores and mycelial fragments by centrifuging are referred to as "diffusates".

The diffusates were examined by various methods for contamination with other microorganisms before being centrifuged. The number of contaminants, chiefly bacteria, varied between 0 and 800 per ml in nine tests.

The antibiotic activities of the crude diffusates were tested by preparing the following dilution series: $n$, $n/2$, $n/4$, $n/8$, $n/16$, or $n$, $2n/3$, $n/2$, $n/3$, $n/4$—where $n$ is the original concentration of the diffusate. Flat blocks of water agar (1 by 5 by 5 mm), seeded with conidia of *Sclerotinia* ($10^3$–$10^4$ per block) were placed in watch-glasses and covered with *c.* 0·1 ml of test liquid. No agar block was used in testing the sensitivity of *Phytophthora*. The test liquid was added to a thick zoospore suspension at an approximate ratio of 3 : 1. Water controls were used in each test series.
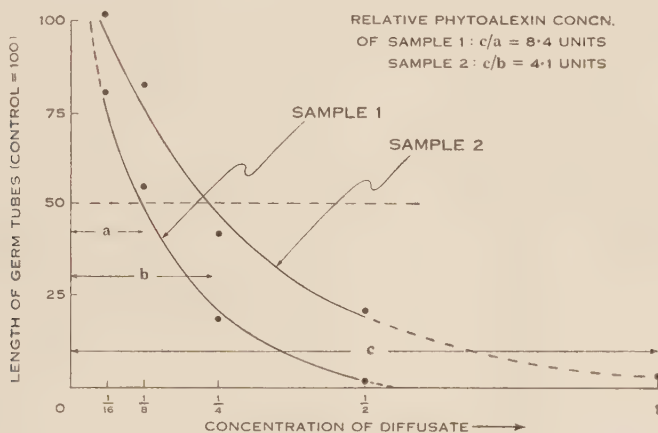


Fig. 1.—Dosage–effect curves demonstrating the mode of calculation of phytoalexin concentration in two diffusates. *Sclerotinia fructicola* was used for inoculation and for bioassay.

In using *Sclerotinia*, antibiotic activity was measured (1) by estimating the germination rate and the growth of the fungus after 18 hr (temp. 23°C) on a —to $++++$ scale where — means no inhibition (=water control) and $+++$ no germination (limit error at 15 per cent.); or (2) by measuring the length of 30 germ tubes lying completely within the focus of the microscope 9–13 hr after starting the test. It was found that alteration of constant temperatures within the range 8–25°C did not affect the sensitivity of the test organism. A critical examination of the reliability of this method was made by Messrs. Dudzinski and McIntyre and is given in Appendix I.

Dosage–effect curves were used to determine, by graphic interpolation, the degree of dilution at which the mean germ tube length was reduced to 50 per cent. of the water control. The reciprocal of this degree of dilution was used as a numerical index of the antibiotic activity of the undiluted diffusate. Figure 1 illustrates the mode of calculation. In addition, this value was used to designate the amount of active units present in a diffusate; the phytoalexin (PA) unit was defined as that amount of PA which, when dissolved in 0·001 ml water, reduces the length of the germ tubes of *Sclerotinia* to 50 per cent. of the control.

Where *Phytophthora* was used as test organism, the standard for the anti-biotic activity of the diffusate was taken to be that degree of dilution at which about 50 per cent. of the zoospores (water control = 100) still reached the stage of germination. Since, as we shall see in a later section, the sensitivity of the zoospores decreases with increasing age, only newly hatched zoospores were used.

Cytological changes which occurred in the infected host cells were studied using hand-sections and staining them with rhodamine B (10 p.p.m.).

## III. Histological Observations

The proportion of *Phytophthora* germ tubes which penetrate into the epidermal cells is relatively high (up to 80 per cent.), and the actual penetration is usually preceded by swelling of the hyphal tips. The actual perforation of the outer wall of the epidermal cell is carried out by a part of the infection peg which is of very small diameter; immediately after perforation, however, when it enters the cell lumen, it expands to a globular organ filled with dense cytoplasm. It is at this stage that growth usually ceases in the type of infections under discussion. In the case of *Sclerotinia*, however, the proportion of germ tubes which enter the cell lumen is considerably smaller than in that of *Phytophthora*. Most of the developing germ tubes grow in close contact with the outer wall of the epidermis, without actually penetrating into the cell. There is here no swelling at the tips of the successful germ tubes such as is seen in *Phytophthora*. At this stage again, soon after penetration, growth of the infection hyphae ceases.

Six to 8 hr after penetration, the cytoplasm of the host cells becomes granular, and simultaneously the nuclei start to swell. After a further 2–4 hr a slight affinity to rhodamine B becomes apparent in the infected cell. This affinity gradually increases and becomes very obvious during the next 10 hr. At this stage in tissues inoculated with *Phytophthora* the staining is restricted to the infected cells (Plate 1, Fig. 1); where *Sclerotinia* is the parasite, however, many cells in the infection area, though showing no evidence of penetration, but only contact with the infection hypha, exhibit stain accumulation (Plate 1, Fig. 2).

After 14–16 hr the epidermal tissue under the inoculation droplets appears to be slowly losing its turgidity. At the same time, the pH decreases significantly within the infected cells, as demonstrated by using di-ethyl red as an indicator. The infected cells turn bright red, the non-infected ones stain yellow. By the use of a variety of indicator stains it was found that the pH drops from 6·3 to around 4·2. In the *Sclerotinia* infections, many cells which have not been penetrated but have only been in superficial contact with the fungus show the red stain when treated with di-ethyl red.

About this same stage in the infection it can be seen that the wall, cytoplasm, and nuclei of the infected cells begin to show an increasing brown pigmentation. The nucleus gradually shrinks, and the whole cell is apparently dying or dead. Since the parasite does not continue to spread after the first incubation period of 24 hr, the inoculated areas now appear to the naked eye as sharply bordered circular surfaces (Plate 2, Figs. 1 and 2).

During the course of the above reactions, the parasite also undergoes significant changes. After an incubation period of 20 hr, the germ tubes also begin to show an affinity for rhodamine B, regardless of whether they have been able to penetrate into the host tissue or not, and about this time also the growth of the parasite in the inoculation drops ceases. No further development of the parasite was observed 24 hr after inoculation. However, if necrotic tissue, which includes the restricted parasite (*Sclerotinia*), is inoculated into plums or apples, growth of the parasite is resumed. This shows that the parasite is still alive.

Summing up the morphological changes which have been found in the epidermis of the seed cavities of bean pods as a result of infection with *Phytophthora* or *Sclerotinia*, we may state that the histological changes observed comply with those which have been found in many other plants after infection with pathogens which interact with the host in a hypersensitive fashion.

## IV. Experimental

### (a) Basic Experiments Demonstrating the Appearance of Antibiotic Factors in Tissue Inoculated with Sclerotinia or Phytophthora

Before describing the inhibitory effects of the diffusates collected from inoculated tissue, it is necessary to mention some experiments designed to show that the tissues do not contain any preformed substance which could be responsible for checking the growth of the pathogens. Three series of experiments carried out for this purpose were:

(1) Slices of tissue consisting of epidermis and subepidermal parenchyma were dissected from pod cavities and treated with (i) a temperature of 110°C for 10 min; (ii) temperatures ranging from 45 to 65°C for 1 hr; or (iii) a temperature of about −10°C for several hours. Immediately after treatment the tissue pieces were inoculated with *S. fructicola*. The fungus developed copiously with luxuriant sporulation. In untreated controls no fungal development took place and local lesions only were seen.

(2) A drop of dense zoospore suspension was added to exudates from tissue which had been pretreated with temperatures of 110 and −10°C. Five hours later, high germination rates and normal germ tube development were observed.

(3) In order to eliminate the possibility that preformed substances may have been destroyed by the treatments used in (1) and (2) above, a third series of experiments was carried out as follows: Parenchyma tissue from the cavities, to which a minimal amount of water had been added, was homogenized, and the resulting pulp was then centrifuged. The supernatant was used immediately in dilution series in a bioassay using *S. fructicola* and *P. infestans* as test organisms for the presence of any inhibitory substances. The germination rates proved normal with both fungi. Only slight inhibition of germ tube growth was observed at concentration $n$.

(i) *Demonstration of the Antibiotic Activity of Diffusates Obtained from Inoculated Bean Pod Cavities.*—After incubation at 20°C for 24 hr, the diffusates collected from cavities inoculated with *S. fructicola* or *P. infestans* appeared brown in colour. After centrifuging, the resulting liquids were perfectly clear and transparent. Their

pH varied from 6·0 to 7·0; their buffering capacity was low. Osmotic concentration varied from 0·5 to 0·6 atm. Surface tension was reduced by 10–14 per cent., in comparison with pure water.

The antibiotic activities of two representative diffusates are shown in Table 1. It can be seen that the undiluted diffusates completely prevent germination, this effect decreasing with dilution to zero at $n/32$. The diffusates obtained from *Phytophthora* inoculations showed a higher activity towards the pathogen, than did the *Sclerotinia* diffusate. The diffusate collected from cavities which had been inoculated with sterile water only, showed no inhibition either of germination rate or of germ tube length.

TABLE 1

ANTIBIOTIC ACTIVITY OF DIFFUSATES TOWARDS THE HOMOLOGOUS FUNGI

Diffusates were collected 20 hr after inoculation. The results were read after an incubation period of 18 hr

| Diffusate Obtained after Inoculation with: | Concentration of Diffusates | | | | | | |
|---|---|---|---|---|---|---|---|
| | $n$ | $n/2$ | $n/4$ | $n/8$ | $n/16$ | $n/32$ | $n/\infty$ |
| S. fructicola* | ++++ | ++++ | +++/++ | + | +/− | − | − |
| P. infestans† | ++ | ++ | ++ | ++/+ | + | − | − |
| Control‡ | − | − | − | − | − | − | − |

*++++, no germination; +++, some germination, very short germ tubes; ++, normal germination rate, length of germ tubes <50 per cent. of control; +, germ tube length about 50 per cent. of control; −, no inhibition.

†++, no germination; +, germination rate about 50 per cent. of control; −, germination as in control.

‡Cavities "inoculated" with distilled water; testing carried out with both *Sclerotinia* and *Phytophthora*.

These results, together with those which have been obtained from experiments with non-inoculated tissue, give strong indications that the inhibitory activity of the diffusates collected from inoculated cavities is due to the post-infectional production of an antibiotic principle.

According to the PA hypothesis it must be expected that tissue from hosts which are susceptible to either of the two pathogens will be unable to produce an inhibitory factor in response to the attack by the congenial parasite. Apricot leaves and slices from fruits of plum, apricot, and apple were tested for their ability to respond to an infection by *Sclerotinia* with PA production. The diffusates obtained showed no antibiotic activity towards the parasite.

Similar experiments were carried out with slices from the tuber parenchyma of a potato variety which is susceptible to the *Phytophthora* strain used in these investigations. Petroleum ether extracts from exudates (see p. 284) collected 24 hr

after inoculation showed no toxicity; after 40 hr incubation, however, a clear inhibitory effect could be observed in the exudate bioassay.

(ii) *The Mode of Action of the Toxic Principle on the Pathogens.*—Spores of *Sclerotinia*, exposed to concentrations of PA which inhibit germination, turn brown and show affinity to rhodamine B. At dilutions which sustain germ tube growth at 5–10 per cent. of the water control, the hyphae are seen to be markedly thicker than those grown in sterile water only; also branching occurs much earlier. After extraction of the toxic principle from the diffusate germ tube growth was markedly better than that of water control. This is obviously a nutritional effect, due no doubt to some nutritional factors in the inoculation drop which have diffused out from the dying host cell. Many hyphae also show curling and twisting in their growth, under the influence of this PA.

TABLE 2

SENSITIVITY OF FRESHLY HATCHED PHYTOPHTHORA INFESTANS ZOOSPORES AND OF ZOOSPORES JUST BEGINNING TO GERMINATE TO A HOMOLOGOUS DIFFUSATE

++, no germination; +, germination rate about 50 per cent. of control; −, germination as in control

| Zoospores | Concentration of Diffusate | | | | | | |
|---|---|---|---|---|---|---|---|
| | $n$ | $n/2$ | $n/4$ | $n/6$ | $n/8$ | $n/12$ | $n/\infty$ |
| Freshly hatched | ++ | ++ | ++ | ++/+ | + | − | − |
| Just starting germination | ++ | ++ | + | − | − | − | − |

*Phytophthora* zoospores respond to higher concentrations of PA with swelling and bursting (plasmoptysis). This process (see Müller 1956, Fig. 4) is preceded by premature contraction of the flagella from the tip, and rapid rounding off of the cell contents. The rapidity with which these changes take place depends on the concentration of the active principle and the temperature. At high PA concentrations and a temperature of 20°C, practically all of the spores have been ruptured after 60 sec. At lower temperatures and at lower PA concentrations it takes more than 30 min to complete this process. Freshly hatched zoospores are more sensitive than those which have already settled down and developed a cell membrane (Table 2). There is no doubt, from microscopical evidence, that PA affects first the structure of the plasmalemma, causing lack of cohesion, and thus rupture of the spores. Occasionally the zoospores do not show plasmoptysis at lethal concentrations and in these cases shrinkage only takes place.

At concentrations around the threshold value of 6 PA units, the inhibition of the spores of *Sclerotinia* has been shown to be only static in effect. If higher concentrations are used, e.g. 10 PA units, then a cidal effect is seen. This was determined by exposing such spores to various concentrations higher than 6 PA units for 24 hr

and thereafter transferring them to sterile water for observation of the germination rate.

### (b) Some Physicochemical Properties of the Active Principle

(i) *Resistance to High and Low Temperatures.*—Samples (1 ml) in sealed, air-tight test tubes were treated at 65°C or at 98°C for 2 hr or at 110°C for 1 hr. There was no reduction in antibiotic effect. Diffusates stored at −10°C for several months retained their full activity.

(ii) *Dialysability.*—The active principle passes through "Cellophane" and collodion membranes. However, the PA yield is reduced by 15–60 per cent. by this treatment.

(iii) *Adsorbability.*—The diffusates lost their activity within 60 min after being mixed with animal charcoal and shaken vigorously. Ordinary filter paper adsorbed 86 per cent. of the antibiotic principle in less than 1 hr. "Cellophane" behaved similarly. Starch, on the other hand, showed no effect.

(iv) *Lipophily.*—The active principle may be extracted from aqueous solutions by means of petroleum ether or *cyclo*hexane (yield from the conventional method, 35–60 per cent.). The residual aqueous fraction possesses no antibiotic effect.

(v) *Sensitivity to Ultraviolet Light.*—The antibiotic activity is lost rapidly if the diffusates are treated with short-wave radiation as transmitted by a Wood's filter.

These results dispel any doubts that a chemical principle or principles is involved which cause the inhibition of the pathogen in the necrotic tissue.

### (c) Experiments on the Dynamics of the PA System

(i) *Relationship between the Concentration of the Diffusates and their Antibiotic Effect.*—A number of dosage–effect curves were subjected to formal analysis. As is generally known, no direct proportionality exists between the concentration and the biological effect of an antibiotic; graphic representation gives curves which deviate to a greater or less extent from the type of a straight line. This is illustrated in Figure 1, which is based on data obtained with two diffusates collected after infection of the seed cavities with *Sclerotinia.*

The relationship is almost certainly disturbed at the lowest concentrations by nutrients in the diffusates and there is some uncertainty about its precise form in the absence of nutrient contamination. In the data examined in Appendix I the curvature of the regression of log length on concentration was convex to the base line and significant.

For this data extrapolation to increasing concentration gave an estimated mean length of 1 per cent. of the control at a PA concentration of 3·65 units and 0·1 per cent. of the control at a PA concentration of 4·66 units. A conservative upper limit based on these and other series for virtually no development would be about 6 PA units. There is, naturally, a dilution effect involved in our tests. This effect depends on the relative volumes of the agar blocks in which the conidia are suspended to the liquids to be tested. The PA values mentioned have therefore to be considered as relative ones.

(ii) *Time Relationship of PA Formation.*—The following problems were investigated:

    (1) How long does it take before PA is detectable in the inoculation droplets?
    (2) How rapidly and to what height does the PA concentration rise in the infection droplets?
    (3) Is the "PA production potential" of the infected tissue exhausted when the maximum PA concentration is reached?

The procedure in test (1) was as follows:

Very concentrated suspensions of *Sclerotinia* and *Phytophthora* were applied to the seed cavities as rapidly as possible at a temperature of 5°C; about 300 seed cavities were used in each case. Fifty inoculation droplets were collected at each of several stated intervals (6, 10, 14, 25, and 29 hr after application of the suspensions) and their activity tested with the homologous parasites. The presence of PA was first detectable after an incubation period of 14 hr in both series of tests. Thereafter, the PA content rose steeply in both series of tests. The increase after an incubation period of 20 hr was insignificant.

In concentrating the samples to one-eighth by the dry-freezing method and re-testing them, PA was found to be present already after an incubation period of 10 hr. The resultant preparation showed an activity of $<5$ and $>3$ PA units. Since about 4 hr elapse before the germination of the conidia, we must conclude from this result that the supply to inoculation droplets is already in progress 6 hr after the parasite has begun to germinate. Since, as it will be shown later on, the host cells retain the accumulated PA to a very considerable extent by "inner" adsorption and release only the excess to the inoculation droplets, the true reaction time may be even shorter.

In our numerous experiments, the PA concentration of the diffusates never exceeded the value of 13 units, even with incubation times of more than 24 hr. The most reasonable explanation for this fact is that PA production is connected with the metabolic activity of the parasite. Since the latter ceases to grow 20 hr after coming into physiological contact with the host tissue, PA production probably also ceases. If after 24 hr a new diffusion gradient was established by removing the inoculation droplets and replacing them by sterile water, and the second series collected and bioassayed, a high activity was obtained.

(iii) *Age of the Host Tissue and PA Production.*—At the very beginning of these investigations it was found that, although the test conditions remained constant, the PA yield might vary quite considerably with the different pod samples. As already stated (Müller 1956), the PA yield depends upon a number of factors, including the external conditions under which the seed pods are kept before being inoculated. The influence of temperature is discussed in detail in Part II of this series (Jerome and Müller 1958). The "physiological" age of the host tissue also is involved here. This was shown by experiments such as the following:

Pods from *Phaseolus* plants raised in a glass-house were divided into three groups according to their stage of development; the seed cavities were inoculated with the same *Sclerotinia* conidia suspension. The resulting diffusates were collected after

24 hr and tested for their PA activity. The results are given in Table 3. The PA production of the older pods (group 3) was found to be more than double that of the younger ones.

The high PA content of the diffusates in group 3 led us to carry out another experiment, using the above method, in order to determine whether the marked inhibition of the parasite in the diffusates of the older seed pods was due to the additional effect of preformed inhibitors. This test gave a negative result.

### (d) *The Antibiotic Character of the Diffusates*

The results obtained from tests with *Phytophthora*-resistant strains of potatoes (Müller and Börger 1940) indicate that the antibiotic principle occurring in diffusates has a wide range of operation. The findings of Kuč *et al.* (1955) and Kuč (1957) point

TABLE 3

PHYTOALEXIN OUTPUT IN RELATION TO THE AGE OF THE PODS

| Group of Age: | Longitudinal Diameter (cm) of: | | Colour of Epidermis of Cavities | Phytoalexin Content of Diffusates (units) |
|---|---|---|---|---|
| | Cavities | Embryos | | |
| 1 (youngest) | <1 | <0·3 | Deep green | 4·7 |
| 2 | 1–1·5 | 0·3–1·2 | Light green | 7·0 |
| 3 (oldest) | >1·5 | >1·2 | Silvery green | 12·7 |

towards the same conclusion; their tests showed that the antibiotic factor obtainable from slices of potato after infection with *Helminthosporium carbonum* is not only effective against this organism, but also against *Fusarium oxysporum*.

In the tests discussed below, the effectiveness of the diffusates was tested in relation to the following fungal organisms: *Colletotrichum lindemuthianum*, *Uromyces trifolii* (uredospores), *Botrytis cinerea*, *Pythium ultimum*, and *Rhizoctonia solani*.

Tests were also carried out to determine whether the diffusate obtained after infection with *Sclerotinia* also possesses antibiotic properties with respect to *Phytophthora*, and vice versa.

Spores which were seeded directly into the test liquid were used in the experiments with the first three organisms. Small blocks of agar (1 by 5 by 5 mm), which were cut from young potato dextrose agar cultures and placed in the undiluted diffusates, were used in the tests on *Pythium ultimum* and *Rhizoctonia solani*. Here, the rate at which the hyphae grew out of the blocks of agar was taken as an index of the antibiotic action of the test liquid.

Results obtained with undiluted diffusates after an incubation period of 18 hr are given in Table 4. They are much as expected: the diffusate derived

## Table 4

DEVELOPMENT OF SEVEN FUNGI IN DIFFUSATES OBTAINED FROM CAVITIES WHICH HAD BEEN INOCULATED WITH SCLEROTINIA FRUCTICOLA OR PHYTOPHTHORA INFESTANS

Data compiled from four experiments

| Diffusate from Cavities Inoculated with: | Phytoalexin* (units) | Fungus | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *S. fructicola*† | *P. infestans*† | *Colletotrichum lindemuthianum*† | *Uromyces trifolii*† | *Botrytis cinerea*† | *Pythium ultimum*‡ | *Rhizoctonia solani*‡ |
| *S. fructicola* | 6·8 | − | − | − | − | − | − | − |
| | 4·0 | ± | − | − | − | − | ± | ± |
| | 2·6 | ++ | − | − | ± | ± | ++ | ++ |
| | 2·0 | ++ | ± | + | ++ | ++ | ++ | ++ |
| | 1·3 | ++ | ± | ++ | ++ | ++ | ++ | ++ |
| | 1·0 | ++ | + | ++ | ++ | ++ | ++ | ++ |
| *P. infestans* | 9·0 | − | − | − | | | | |
| | 4·5 | ± | − | − | | | | |
| | 2·25 | ++ | − | + | | | | |
| | 1·12 | ++ | ± | ++ | | | | |

*Refers to reaction of *S. fructicola*.

† −, no germination; ±, germination of only a few spores; +, germination rather strong, but significantly less than in water control; ++, germination as in control.

‡ −, no outgrowth of hyphae; ±, length of outgrown hyphae less than 25 per cent. of control; +, length of hyphae 25–50 per cent. of control; ++, length of hyphae 50–100 per cent. of control.

from tissue infected with *Phytophthora* completely inhibited the germination of the *Sclerotinia* conidia; the diffusate obtained from tissue infected with *Sclerotinia* had the same effect with *Phytophthora* zoospores. Germination was likewise inhibited in the case of *Colletotrichum lindemuthianum*, the fungus responsible for bean anthracnose. This organism, as shown by another series of tests, appears to be slightly more susceptible than *Sclerotinia*.

The results obtained with *Pythium* and *Rhizoctonia* merit closer consideration. No signs of growth were visible with both fungi after an exposure period of 18 hr. The situation altered during the next 24 hr, however; numerous hyphae had meanwhile grown out of the blocks of agar. The rate of growth was considerably less than in the case of the water control, but finally the two fungi succeeded in growing completely through the test liquid. Further investigations must be carried out before a satisfactory explanation for this phenomenon can be given, i.e. whether there is an "adaptation" of these two fungi to the antibiotic factor or whether the toxic principle is destroyed by the metabolic activity of the fungi.

### (e) Influence of Nutrient Concentration and of pH of the Substrate on PA Activity towards Sclerotinia

The *Sclerotinia* fungus is readily cultivable, a feature which enabled us to investigate the extent to which the PA sensitivity of the parasite and thus the clinical result of an infection is affected by the content in the host tissue of substances which the parasite is able to use as nutrients. It could be possible, for example, that an abundant supply of nutrients may reduce the PA sensitivity of the parasite and thus increase its virulence. Similar ideas have recently been voiced by certain authors who have treated "host–parasite relationships" primarily as a nutritive–physiological problem. Lewis, for example, states in a recent paper (1953): "We find substances that enhance and substances that inhibit the activities of parasites. We find also that enhancement or inhibition depends on the nutritional environment in which the substances operate. Is it not readily conceivable that these or similar factors determine the success or the failure of a parasite in its host?"

From a methodological point of view, too, it seemed desirable to ascertain whether an antagonistic or a synergistic relationship exists between the PA content and the nutrient content of the substrate. It is true that we investigate the PA sensitivity of the organisms, which give rise to the PA formation, in a substrate to which no nutrients are added. However, the "natural medium" in which the interaction between parasite and host cell occurs is very different from the conditions under which the PA sensitivity is tested *in vitro*. If, therefore, substances which may be used as nutrients by the parasite do actually influence its PA sensitivity, it becomes extremely difficult to apply the results of our *in vitro* tests to *in vivo* conditions.

The following experiments were carried out: *Sclerotinia* conidia were suspended in agar blocks at increasing nutrient concentrations. As "sources of nutrients", boiled filtered juice from potato tubers, plum fruits, or from the parenchyma of bean pods were used. Small cubes of agar, about 25 mm³ in size, were taken from each concentration and placed in test solutions of varying PA content.

The result of an experiment, in which juice from the parenchyma of bean pods was used as nutrient, may be taken as a typical example. As we see from the results in Table 5, the added nutrients had no significant influence upon the PA sensitivity of the parasite.

Filtered juice from potato tubers or plums was used in certain other tests. Here, too, the results proved to be negative.

In the above experiments filtered juices, which contained practically no solid cell constituents or proteins, were used. If non-filtered juices were used, i.e. those containing abundant proteins and cell particles, a slight increase of the germ tube length was noted with a PA concentration corresponding to 2·65 units (see Table 5).

TABLE 5

DEVELOPMENT OF SCLEROTINIA AT DIFFERENT PHYTOALEXIN CONCENTRATIONS AND NUTRIENT CONTENTS

Homologous diffusate was used. Results refer to the relative germ tube length

| Nutrient and Treatment | Nutrient Concn. (%) | Concentration of Phytoalexin (units) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 10·6 | 5·3 | 2·65 | 1·33 | 0·67 | 0·00 |
| Bean sap, boiled and filtered | 60 | 0·0 | 0·0 | 14·5 | 41·6 | 72·9 | 100 |
| | 15 | 0·0 | 0·0 | 15·4 | 34·0 | 69·0 | 100 |
| | 3·75 | 0·0 | 0·0 | 17·3 | 40·3 | 60·5 | 100 |
| Bean sap, boiled only | 60 | 0·0 | 0·0 | 21·0 | 55·1 | 70·2 | 100 |
| | 15 | 0·0 | 3 | 22·0 | 44·1 | 67·6 | 100 |
| | 3·75 | 0·0 | 0·0 | 18·9 | 40·3 | 65·6 | 100 |
| No bean sap (control) | 0·0 | 0·0 | 0·0 | 13·2 | 40·2 | 69·4 | 100 |

Since, as mentioned on page 280, the acidity of the host cells clearly increases soon after the PA formation commences, the pH of the substrate was next considered as an additional factor having a possible influence on PA activity. It is known, too, that the effectiveness of many antibiotics, such as streptomycin, is partly determined by the pH of the substrate. A series of experiments was therefore carried out, as described in the following paragraph.

Solutions at six different degrees of dilution were prepared from a diffusate obtained from tissue infected with *Sclerotinia*. These were mixed with phosphate buffer solutions (0·2M) at pH values of 3·5, 4·5, 5·0, 6·0, 7·0, and 8·0 in the ratio of 1 : 1. Pure water and buffer solutions served as control. *Sclerotinia* was used as test organism. The result of a typical test series shows that the pH of the substrate exercises no effect upon PA activity in the range between pH 4·0 and 7·5.

## (f) Sorption Phenomena

At the commencement of our investigation there were many indications that the amounts of PA occurring in the diffusates represented only a part of the total PA formation. This hypothesis was supported by the following experiment: Seed cavities were treated with a diffusate containing 3·7 PA units. The latter was recovered after an incubation period of 20 hr and tested for its PA activity; the result showed a reduction in the antibiotic effectiveness to <0·5 units.

If we apply this result to the conditions obtaining in the host tissue, we must postulate that large PA amounts given off by the infected cells to the adjacent cells are immediately fixed and accumulated by the latter. Such a mechanism would therefore exclude free diffusion of the "excess" amounts of PA into the surrounding host tissue.

Further experimental data were needed to confirm this hypothesis. Several problems had to be answered:

(1) What is the rate at which the "free" PA present in the diffusates is fixed by living tissue?

(2) Does total binding of the free PA occur?

(3) Is dead host tissue also capable of binding PA?

The following procedure was adopted in the experiments carried out in connection with the above problems:

Tissue taken from the parenchyma of bean pods was placed in test tubes, which were then filled with sufficient amounts of a standardized PA solution to ensure that the samples of tissue were completely covered by the solution. The test tubes were then transferred to a shaking apparatus. The liquid was tested for its PA activity at specified intervals. Since the volumes of the particles of tissue and of the diffusates used were known, it was possible to determine the extent and the rate of PA fixation by the particles of tissue from the decrease in the antibiotic effectiveness of the supernatant liquid.

From the diagram in Figure 2 (curves A and B), we see that more than 80 per cent. of the PA contained in the diffusate had already been fixed by the living tissue after the first 10 min. Since no further decline in the PA activity in the supernatant liquid after this time was apparent, a stable equilibrium between the amount of PA bound by the tissue and the "free" PA must have been established within 10 min.

The same experiment was carried out with tissue which had been killed by heating to 100°C. The result is given in Figure 2 (curves C and D). It shows that the dead tissue was also capable of binding considerable amounts of PA. However, the rate at which the liquid lost its antibiotic effectiveness in the test with dead tissue did not appear as great as with the living tissue. The final values for PA fixation were also lower.

The results of these tests may also provide a satisfactory explanation for the apparent decrease in the PA sensitivity of the parasite in unfiltered cell sap, as described on page 289. Part of the PA present in the test liquid is evidently fixed by adsorption by the abundant proteins and cell fragments, and the PA concentration

thereby reduced. However, the possibility must be considered that rather than being only adsorbed the PA may be inactivated.

(g) *Phytoalexin Formation in other Hosts than* Phaseolus vulgaris *after Infection with* Sclerotinia *or* Phytophthora

We have hitherto been concerned solely with the antibiotic activity of diffusates obtained from infected *Phaseolus* tissue. The question that now arises is whether the reaction mechanism described above has a wider significance. Three other host plants were investigated to determine whether the local lesion reaction is associated with PA formation in these particular cases also.
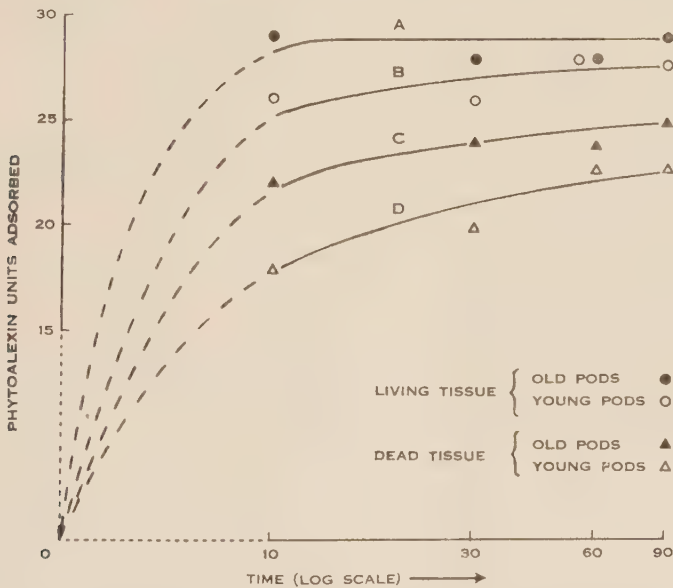


Fig. 2.—The amount of phytoalexin adsorbed to the host tissue after different intervals.

The test plants selected were *Pisum sativum*, *Vicia faba*, and *Capsicum annum*; *Sclerotinia* and *Phytophthora* were again used as parasites, and the inner epidermis of the fruits was again used as host tissue. It was found that these three plants also respond to infection by local lesions. After an incubation period of 24 hr the PA content in the infection droplets was found to be relatively high. The PA activity of the crude diffusates corresponded approximately to that found in the tests with *Phaseolus*. It was possible, in all cases, to separate the antibiotic principle from the crude diffusates by means of petroleum ether extraction.

These plants were likewise tested in the manner described above (see p. 281), in order to ascertain whether the antibiotic activity of the diffusates is due to the exosmosis of preformed inhibitors from the dying host cells. The behaviour of the germinal hyphae of *Sclerotinia* upon tissue previously treated at low or high temperatures (−10 or +50°C) lends no support to such a hypothesis. In these cases, too,

there is therefore not the slightest doubt that the inhibition of the parasite in the affected tissue is due to the action of antibiotic factors which first develop in the course of the interaction between parasite and host.

## V. Discussion

The main purpose of the investigations described in Section IV was to determine whether the inhibition of the parasite in the local lesion tissue is due to the post-infectional formation of antibiotic agents. The results of the basic experiments may be summarized as follows:

(1) The epidermis of the seed cavities of *Phaseolus vulgaris* which produces local lesions in response to infection with *Sclerotinia fructicola* or *Phytophthora infestans*, releases—after a relatively brief incubation period—a principle (PA), which exerts an antibiotic effect upon the parasites.

(2) Under the particular conditions of these tests, the effective principle occurred in amounts which were quite sufficient to totally inhibit growth of the parasites.

(3) This principle was not detectable in healthy tissue.

(4) There was no formation of an antibiotic factor (or only after a relatively long incubation period) in hosts, which are susceptible to the above-mentioned parasites.

(5) The principle separated from the host tissue possessed physical and chemical properties which exclude all doubts regarding the existence of biologically active chemical compounds in the diffusates.

(6) A basic condition for its development is that the host tissue should be physiologically "normal".

These results may be taken as confirmation of the hypothesis that the inhibition of the parasite in the local lesion tissue is due to the activity of an antibiotic principle, which does *not* occur "preformed" in the host cell, but which owes its origin to an interaction between the host and the parasite. In view of this finding, there can be no further doubt that—in the present case—the changes occurring in the reactive tissue must be regarded as defensive reactions.

Supplementary tests were made with *Pisum sativum*, *Vicia faba*, and *Capsicum annum*. Here, too, infection with *Sclerotinia* or *Phytophthora* resulted in the development of local lesion and, at the same time, in abundant PA formation. Kuč *et al.* (1955, 1956) and Kuč (1957) have also demonstrated the post-infectional formation of an antibiotic factor following the infection of potato tubers with *Helminthosporium*; it follows, therefore, that a more general significance must be attached to the defensive mechanism which is the subject of the present discussion.

Given the present state of our knowledge of this matter, however, it would be premature to attempt to interpret every local lesion reaction in the light of the PA concept. It is conceivable, for example, that preformed inhibitors only begin to take effect after the structure of the plasmalemma of the host cell has been destroyed by metabolic products of the parasite, and the inhibitors thus come into direct

contact with the hyphae of the parasite for the first time. In this case, too, inter-action would result in the death of *both* partners. This idea has been proposed by previous workers who found inhibitory factors in susceptible hosts as well; for instance, Newton and Anderson (1929) investigated the phenolic compounds in eight wheat varieties resistant in varying degrees to rust and found no correlation between content of phenolic compounds and rust resistance. Scott, Millerd, and White (1957) employ a similar concept in an article on the local lesion reaction occurring in barley after infection with non-virulent *Erysiphe* strains. They also postu-late such a reaction mechanism because they had found that susceptible plants also contain a preformed phenolic factor, which has an inhibiting effect upon the parasites. Unfortunately, no information is given as to whether this factor is present in concentrations sufficient to check the parasite *in vivo*.

Further tests were carried out in conjunction with the basic experiments, primarily for the purpose of studying the mechanism of the local lesion reaction.

The antibiotic action of the PA derived from *Phaseolus* is non-specific. This agrees with the findings of Kuč and co-workers, who state that the antibiotic sub-stance isolated from potato slices infected with *Helminthosporium* is also effective against *Fusarium oxysporum*. The non-specific nature of PA, as postulated by Müller and Börger (1940), is thereby confirmed.

It may seem difficult, at first glance, to reconcile the non-specific character of PA with the fact that strongly marked specialization exists with many host-parasite combinations. With rust infections, for example, the mere presence or absence of a single gene, on the side of either the host or the parasite, is sufficient to determine whether the interaction between the two partners will result in local lesions. Catcheside (1951) does not hesitate, therefore, to draw a parallel between the mutual relationship between the genes of the host and the parasite and the well-known antigen–antibody reaction from the field of animal pathology. However, there is no fundamental difference here. If, following Müller (1950), we extend our observations to all theoretically conceivable host–parasite combinations, then the situation is reversed. For we find that the characteristic of specificity does not pertain to the local lesion reaction, but rather to its opposite, i.e. the mutual tolerance between host and parasite. However, this weakens the argument advanced previously against the non-specific nature of the factor which causes the inhibition of the parasite in the local lesion tissue. There is another argument which supports the idea that the end-result of the interaction between host cell and parasite need not necessarily be a specific one: there is no doubt that a "local lesion reaction" on the one hand, and a "mutual tolerance" of the two partners on the other are the extremes of the same category of interaction, reflecting differences only in degree. This quantitative approach is justified by a number of facts: In the first place, the reaction of intermediate host types may change with changes in environmental conditions and age of the host, towards the one or the other extreme. Furthermore, it has been shown that the rate of reaction of the host has a strong impact on the clinical outcome of the interaction between the two partners; the greater the inter-action rate, the earlier the pathogen's growth is checked (Müller and Börger 1940; Müller 1953). In view of the fact that the principle inhibitory to the pathogen is

active against other organisms also, we are compelled to the conclusion that the specificity of an interaction (actually that of mutual tolerance between pathogen and host) rests on particular factors, both of host and pathogen, which meet in the infected host cell and determine the rate with which the non-specific principle inhibitory to the pathogen appears at the infection site.

Thus, in the light of our considerations, there is no disease resistance as such, but only the host being able to render the parasitized tissue inhospitable to the pathogen with due speed. This would mean in terms of our PA concept: there should be a threshold concentration of PA which must be reached within a certain period of time; otherwise the pathogen will continue to spread in the host tissue.

This concept of the nature of "active" or "protoplasmic" resistance is supported by experimental results obtained in this work: (1) the interaction starts within a few hours of the pathogen contacting the host cells, and (2) a few hours later, the PA produced per unit volume of the reacting tissue exceeds by far that amount at which no further growth of pathogen occurs. Further evidence is given in Part II (Jerome and Müller 1958).

Further results, which may be regarded as decisive for the causal understanding of the mechanism upon which the local lesion reaction in our test objects is based, are as follows:

(1) A high affinity exists between the antibiotic principle and the host tissue.

(2) The amount of PA eventually obtained is partly determined by the age of the host tissue.

(3) The PA's found to date possess lipophilic properties.

In the interpretation of our test results, it should be remembered that, when our method is used, the conditions under which the interaction between parasite and host tissue takes place are very different from those under which it occurs in the natural environment. The ratio between the volumes of infection drops used and of the host tissue is "unnaturally" high; the diffusion equilibrium between the PA concentration in the infection droplet and in the reactive tissue must therefore necessarily occur at a relatively late stage. This implies, in any case, that where parasite and host tissue are in direct contact, the PA concentration must be considerably higher than in the inoculation drops. Furthermore, the diffusion equilibrium is delayed by adsorption of PA by the reacting host cell. That is, we must conclude from our experiments that "free" PA only becomes available for release to the infection drops after the host cell has become "saturated". In addition, the competitive action on the part of the non-infected neighbouring cells must also be taken into account; these cells, in their turn, fix large amounts of PA.

It has already been mentioned that the release of PA to the infection drops was found to be in operation about 6 hr after germination of the parasites. From the above observations it appears that the first amounts of PA accumulate considerably earlier within the reactive cells. This also corresponds to the fact that the development of the parasite does not, as a rule, pass beyond the formation of short infection hyphae.

As already stated in an earlier publication (Müller 1956), the interaction product exercises a toxic effect, not merely upon the parasite, but upon the host cell as well. A preparation, semi-purified by dialysis, with a PA activity of about 16 units produced increased rhodamine B affinity in the treated epidermal cells. At the present time, unfortunately, it is impossible to decide whether the principle acting upon the parasites is identical with the one which causes the necrotic changes in the host cells. Only the chemical identification of the active principles can supply an answer to this problem.

As shown in a recent paper (Müller 1958), the PA released per unit volume of infected host tissue is extraordinarily high. Under the conditions of our experiments, it is more than a thousand times the amount required to check the parasite at the site of infection. If we consider that in the diffusates only those amounts of PA can be detected which have escaped fixation by the host tissue, we see that the total PA output must be estimated at an even higher figure than that quoted in the above-mentioned work.

The high adsorption characteristics of the active principle may also be important in another respect for a causal understanding of the local lesion reaction. Many observers have stressed the fact that it is not only the cells in direct contact with the parasites, but also the neighbouring cells as well—often merely the adjacent membranes with the corresponding protoplasm—which reveal histological changes corresponding to those observed in the case of the infected cell. This indicates that a factor is given off, from the cells which interact directly with the parasites, to the neighbouring cells where it is fixed, and that the changes are caused by this factor. This probably also applies to the amounts of free PA which are released by the reacting cell to its surroundings after saturation is complete. Consequently, as our sorption tests indicate, free diffusion through the adjoining tissue must be impossible, and a zone of high PA content is formed, which surrounds the seat of infection like a protective wall. This presumably prevents the parasite from escaping from the seat of infection into the adjacent tissue. The fact that the formation of PA begins anew once a fresh diffusion gradient has been created (see p. 285), may also be regarded as an additional safety device.

The fact that the isolated antibiotic factors possess lipophilic properties indicates that free PA is primarily adsorbed to the lipoid particles of the host cells. Since the lipoids, owing to their surface activity, occur preferentially in the plasmalemma, the neighbouring cells probably adsorb the greater part of the free PA at their surfaces. However, this is the place where the hyphal tips of the pathogen come into contact with the neighbouring cell first.

Our knowledge of the antibiotic activity of PA fixed by adsorption is still very far from complete. However, it seems reasonable to assume that destruction of the plasmalemma by the metabolic products of the parasite results in the release of previously adsorbed amounts of PA, which consequently recover their antibiotic effectiveness. This process of alternate fixation and liberation of PA may perhaps explain why the parasite—provided it is not inhibited by the primarily infected cells—encounters increasingly high concentrations of PA, until it is finally obliged to cease growing altogether.

As already mentioned in an earlier report (Müller 1956), the mechanism under-lying the formation of PA is inhibited by brief preliminary treatment of the host tissue at high, but not lethal, temperatures. The relationship of the PA formation to the physiological state of the pod tissue was further confirmed in these experiments by the fact that the PA production potential increases in the course of the individual development of the pods. This phenomenon appears to be similar to the well-known increase in the sensitivity of many plants and thus in increasing "reactive resistance" to fungal parasites during the individual development of the host plant.

Although it is now possible to offer a reasonably satisfactory solution to the cardinal problem studied in these investigations, i.e. the demonstration of the principle inhibiting the parasite in the infected tissue, the work as a whole gives the impression of incompleteness; in the first place, because the query as to the chemical structure of the active principle is left unanswered. The principal aim, however, was to separate from the interacting host tissue the factor which is the actual cause of the inhibition of the parasite and study some biological aspects of the problem; the question of its chemical structure was thus essentially a secondary problem. Most earlier writers on this subject endeavoured, by the use of conventional methods of analytical chemistry, to demonstrate a presumed factor which makes a resistant plant resistant. In the majority of cases, the plant was used in the uninfected state. It will be evident from the results discussed above why the goal could not be reached in this way: it is completely impossible to trace this particular factor in healthy plants, simply because it only appears at concentrations sufficient to stop the pathogen *after* physiological contact between host and parasite has been established. The reverse procedure was adopted in our investigations: whilst "sparing" the physiological structure of the interacting partner as far as possible, the principle inhibiting the parasite was separated from the host tissue and tested quantitatively for its antibiotic effectiveness.

The most important tasks facing us now are to determine the chemical structure of the active principle, which the present author called "phytoalexin" almost 20 years ago and later (Müller 1953) defined as "an antibiotic which is the result of an inter-action of two different metabolic systems", and to identify the factor which, released by the parasite into the host cell, gives rise to the formation of phytoalexin. Thanks to modern biochemical methods, in conjunction with the special methods of pro-duction and testing employed in our investigations, it should not be difficult, once the chemical structure of these two factors is known to set up a theoretical model of the mechanism by which the plant is enabled to defend itself *actively* against potential parasites.

## VI. Acknowledgments

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## Appendix I

### BIOLOGICAL ASSAY USING LENGTH OF GERM TUBES AS THE MEASURE OF RESPONSE

By M. L. Dudzinski* and G. A. McIntyre*

Data was available on individual lengths of 30 germ tubes of *Sclerotinia fructicola* for successive levels of twofold dilutions of two samples (A, B) of diffusate on each of two days. Between these assays the samples were stored at $-10°C$ and there was little likelihood of any deterioration taking place in the interval. During development of the hyphae the temperature control was imperfect but all levels of dilution for each sample together with the corresponding control on each test were subjected to the same conditions. This implies that for comparative purposes the development at the various levels of dilution must be expressed in terms of the corresponding control. Analytically this implied the use of logarithms of germ tube length in fitting regression curves. This transformation is also favourable in the sense of making the variances within treatments more uniform.

To reduce the influence of outlying observations the means of six successive germ tube lengths were taken as the primary entries into the analysis, giving five means per level. The variances of the logarithms of these means were examined for homo-

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geneity between treatments. The variance was greater for treatments with low than with high means. The trend was linear and variances for particular means were estimated from a fitted line. The reciprocals of the variances were used as weighting factors in the subsequent estimate of constants by least squares.
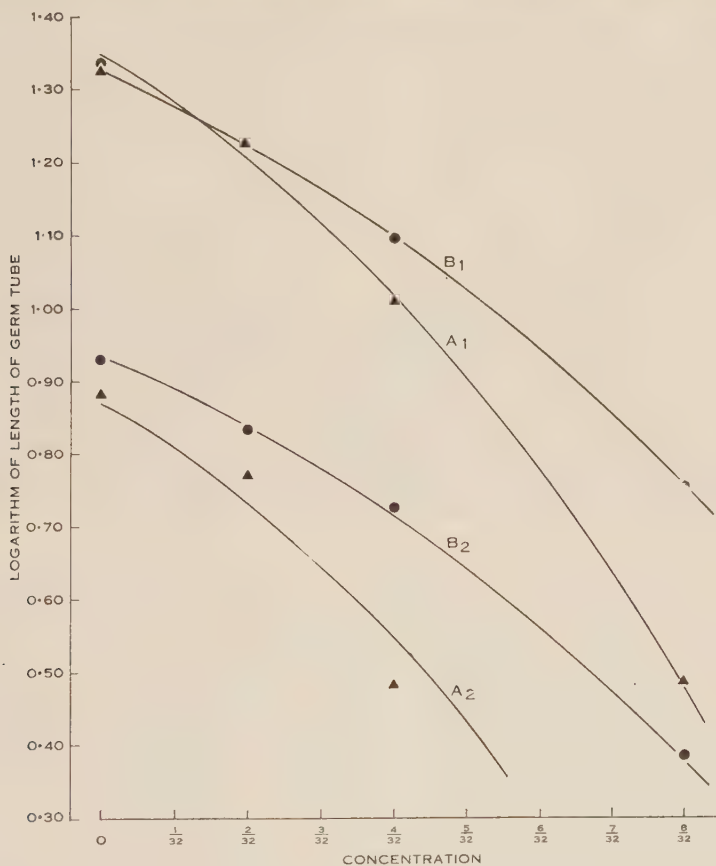


Fig. 3.—Relation between log germ tube lengths and concentration of diffusates for two samples on each of two days.

The form of regression equations used was

First day   (A$_1$)                $Y_1 = a_1 + b_1 x + b_2 x^2,$
            (B$_1$)                $Y_2 = a_2 + b_1(kx) + b_2(kx)^2,$
Second day (A$_2$)                $Y_3 = a_3 + b_1 x + b_2 x^2,$
            (B$_2$)                $Y_4 = a_4 + b_1(kx) + b_2(kx)^2,$

where $Y = $ log (germ tube length) and $x$ is the concentration expressed as a fraction of the initial concentration.

The first and second equations and likewise the third and fourth are related on the assumption that the second preparation can be regarded as a dilution of the first so that its effect can be represented by the same expression but with a change

in concentration $x$ by factor $k$. It was assumed $k$ would remain constant from the first to the second day. The equations imply that a vertical displacement will bring $A_2$ into coincidence with $A_1$, and $B_2$ with $B_1$. With a common ordinate at zero concentration the ratio of the abscissa, B to A, for any hyphae length is the relative concentration $k$.

This set of equations was fitted to the data by least squares using an iterative procedure. The final iteration gave the equations

$$Y_1 = 1{\cdot}3475 - 1{\cdot}7728x - 6{\cdot}8096x^2,$$
$$Y_2 = 1{\cdot}3247 - 1{\cdot}7728(kx) - 6{\cdot}8096(kx)^2,$$
$$Y_3 = 0{\cdot}8722 - 1{\cdot}7728x - 6{\cdot}8096x^2,$$
$$Y_4 = 0{\cdot}9462 - 1{\cdot}7728(kx) - 6{\cdot}8096(kx)^2.$$

The data and fitted curves are presented in Figure 3.

The factor of relative concentration, $k$, is $0{\cdot}739$ with a standard error of $0{\cdot}034$, which is a satisfactory level of precision. The value of $\chi^2$ for goodness of fit was $6{\cdot}94$ for seven degrees of freedom so that there is no statistical evidence that the model is at fault.

The value of $x$ at which the length is half the control is the value corresponding to $Y$ less than the control by $0{\cdot}301(\log 2)$. Defining this as the unit concentration of phytoalexin the values of $x$ for A and B are $0{\cdot}1171$ and $0{\cdot}1585$. The strength of the undiluted samples is then $8{\cdot}53$ and $6{\cdot}30$ phytoalexin units.

The ratio of $b_2$ to $b_1$ is almost 4. The curves in this instance could be made linear by a transformation $z = (x + 4x^2)$. The data of this analysis are almost certainly disturbed by the effects of nutrients in the diffusates and it is possible that with nutrients eliminated that either a transformation would not be necessary or one could find a standard transformation of this form which would give a linear relation between log length and the transformed concentration. This would simplify the statistical procedures and calculation of errors of estimates.

STUDIES ON PHYTOALEXINS. 1
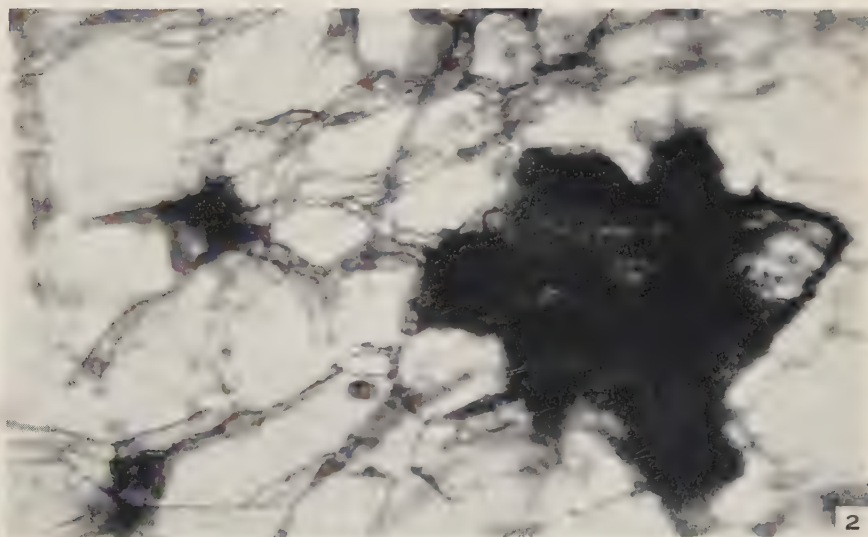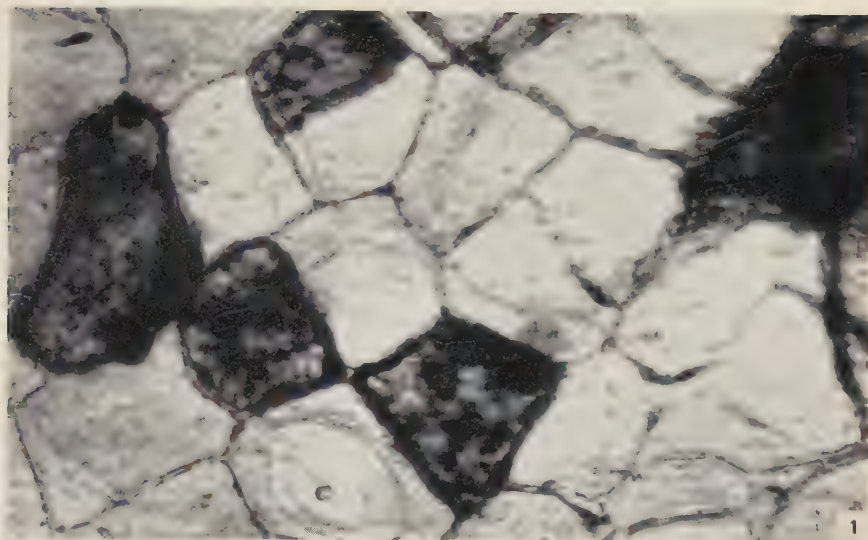


Host cells infected by *Phytophthora infestans* (Fig. 1) and *Sclerotinia fructicola* (Fig. 2).
Incubation period: 20 hr at 20°C. Stain used: rhodamine B (10 p.p.m.). × 470.

STUDIES ON PHYTOALEXINS.  I
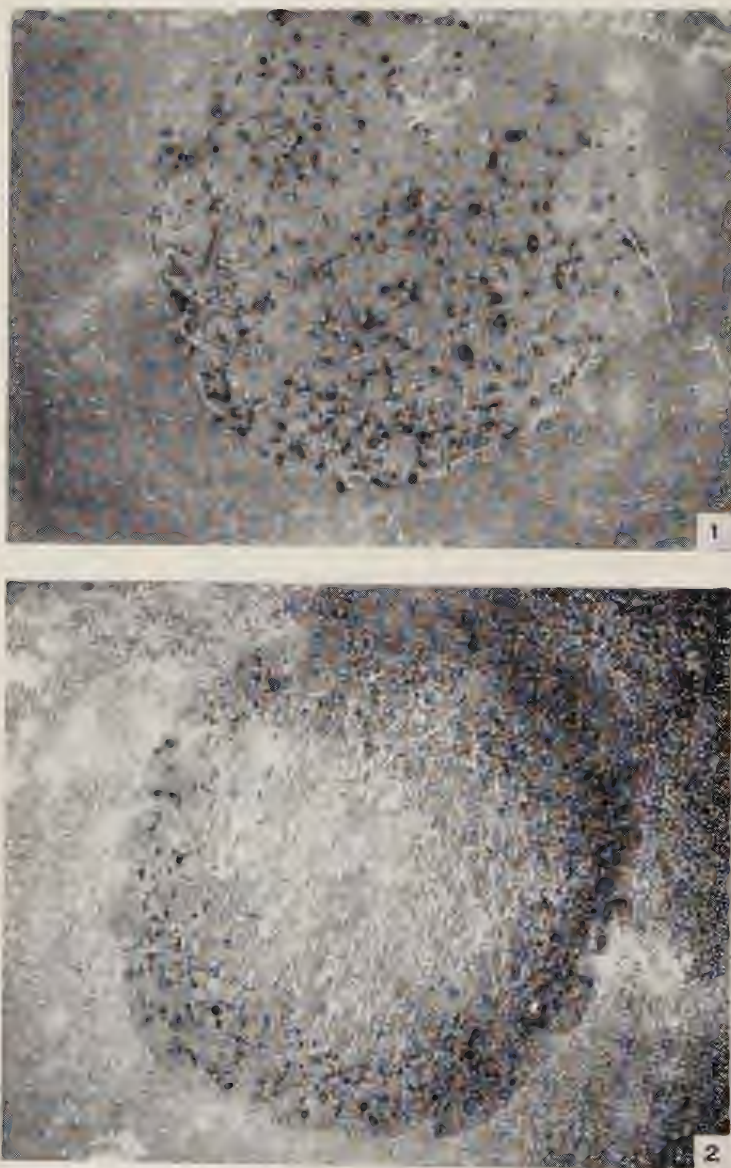


Areas of seed cavities inoculated with *Phytophthora infestans* (Fig. 1) and with *Sclerotinia fructicola* (Fig. 2).   Incubation period: 48 hr at 20°C.   × 15.

# STUDIES ON PHYTOALEXINS

## II. INFLUENCE OF TEMPERATURE ON RESISTANCE OF PHASEOLUS VULGARIS TOWARDS SCLEROTINIA FRUCTICOLA WITH REFERENCE TO PHYTOALEXIN OUTPUT

By S. M. R. Jerome* and K. O. Müller*

*Summary*

(i) The resistance of pods of *Phaseolus vulgaris* towards the parasites *Sclerotinia fructicola* and *Botrytis cinerea* is shown to be affected by the temperature environment before inoculation.

(ii) At the critical temperature of 44°C for 2 hr, the loss of resistance is seen to be a reversible process. Recovery takes place within 3 days, when stored at a temperature of 20°C. The storage temperature after heat-conditioning is important for the recovery process.

(iii) The output of the post-infectionally produced defensive substance—phytoalexin—is likewise affected by pre-inoculation conditioning at various temperatures. Under all conditions there is a close correlation between the clinical behaviour of the pods and phytoalexin production.

(iv) Respiration rate as shown by oxygen uptake in air is affected by the preconditioning treatment with high non-lethal temperatures. There appears to be no direct correlation between the oxygen uptake and the clinical behaviour or phytoalexin output, though there is an obvious connection.

(v) Practical applications of these results are considered: the effect of climate on the state of resistance of hosts to particular parasites; the optimal conditions for storage of fruit and vegetables.

## I. INTRODUCTION

It is generally agreed that by altering the environmental or cultural conditions of plants before or after inoculation it is possible to modify their resistance to a specific pathogen. Treatment with different temperatures has been an important aid in demonstrating this fact. Most of the earlier investigations were concerned with the influence of post-inoculation changes of temperature (Stakman and Lambert 1928; Waterhouse 1929; Johnson 1931, to mention only a few). Comparatively little has been done on the effect of conditioning the host by temperature changes before inoculation. Salmon (1904, 1905) first showed that it was possible to infect normally immune varieties of plants by pretreating them with heat or anaesthetics. Since then, Gradinaroff (1943), Kassanis (1952), and Yarwood (1956) have recorded alterations in the resistance of various hosts towards fungal parasites or viruses brought about by preheating the host tissue. Although this phenomenon has been observed and recorded, little attempt has been made to explain it, or account for it. Müller (1956), describing the post-infectional production and properties of antibiotic substances which he named phytoalexins, showed that by treating bean pods

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for 90 min at 41–42°C he could reduce their resistance to *Sclerotinia fructicola* and their phytoalexin output. Müller attributed the decrease of resistance in the preheated pods to a blocking of the phytoalexin production system.

The investigations to be described in this paper are concerned with such changes of resistance, and especially with the mechanism underlying these changes, in pods of *Phaseolus vulgaris* towards the parasites *S. fructicola* and *Botrytis cinerea*.

## II. MATERIALS AND METHODS

Beans used in these experiments were grown at Dickson Experiment Station, A.C.T. Because material of uniform quality was necessary for respiration studies, beans for this purpose were produced in the greenhouse under optimal conditions. The pods were harvested, surface-sterilized with 0·1 per cent. mercuric chloride, and prepared for the various treatments by splitting longitudinally or halving laterally with a sterile scalpel and enclosing each half in a sterile tube closed with a cotton-wool stopper. In some cases the pieces tubed for treatment consisted only of a single seed cavity.

For investigating the effect of high temperatures a thermostatically controlled water-bath was used. The same N.S.L. thermometer was used throughout. The tubes containing the pod pieces were kept in the bath at constant temperature for 2 hr. The level of the water was such that the tubes were three parts immersed, and thus the enclosed pods were wholly immersed. After heating, a sample was taken from each treatment for immediate inoculation and the remainder of the treated bulk stored at the appropriate temperature (whether 20 or 2°C) in wire baskets covered with plastic squares to reduce drying.

For the effect of low temperatures pods, prepared as above, were stored in tubes at the required temperatures for continuous periods. Samples were taken at intervals.

After treatment, samples required both for clinical observations and for phytoalexin (PA) production studies were prepared as follows: The pods removed from tubes were again split longitudinally with a sterile knife, thus exposing the seed cavities. The seeds were removed and the cavities with an unbroken epidermis were set out in pie-dishes lined with moist filter paper. The cavities were then inoculated with drops of spore suspension applied by capillary pipette. Dishes were covered with glass sheets and kept at 20°C. Unheated beans (controls) were set up alongside each treated sample. The spore suspensions were prepared from pure cultures of *S. fructicola* or from *B. cinerea*, grown on potato dextrose agar. The latter was previously isolated from an infected bean pod.

The drops were recovered 24 hr after inoculation, centrifuged in bulk, and the PA activity determined by bioassay using *S. fructicola* as test organism, according to the technique described by Müller (1956, 1958b). The dishes were left for a further 3 days at 20°C when clinical response to infection was assessed.

The bases of assessment for clinical response were three, viz. local necrosis of host tissues, fructification of fungus and complete destruction of host tissue, or intermediate. This latter denoted an infection where only a small amount of mycelium could be detected with little or no sporulation. A numerical assessment

assigned to these was: necrosis = 0, fructification = 1, intermediate = 0·5. For certain purposes of comparison these numerical assessments were percentaged and related to the full number of cavities inoculated, which represented unity. The PA assessment was made from graphical representations in which PA activity of the control was taken as 100 per cent. and each sample from treated material was related to its own control for assessment. The PA was also assessed in PA units. By definition, a unit of activity is that concentration of the inhibitory principle present in a PA solution which reduces the growth of the germ tubes of *S. fructicola* to 50 per cent. of the control (Müller 1958b).

All measurements of respiration were made in a Warburg apparatus at 20°C. Oxygen uptake in air ($Q_{O_2}$), expressed as $\mu$l of $O_2$ per 100 mg dry weight per hr at 20°C, was measured in pods preheated at 44°C for 2 hr (i) immediately after treatment, and (ii) 3 days after treatment (storage at 20°C). Unheated pods were used as controls. The samples to be used, after the above treatments, were split longitudinally and prepared as above. Half pods were placed in moist chambers, inoculated with *S. fructicola*, and incubated for 24 hr at 20°C for PA production and clinical observations. The corresponding halves were cut into pieces consisting of single cavities only. Five pieces of consistent size, selected at random, with a total dry weight of approximately 200 mg were used in each Warburg flask. Since the number of pieces per flask was always the same, the area of cut surface was also approximately similar in each case. Readings were taken over a period of 30 min after 15 min equilibration time.

### III. Experimental Results

#### (a) *Influence of Temperature on Clinical Behaviour of Pods*

(i) *High Temperatures.*—Pods heated at 50°C for 20 min were inoculated with *S. fructicola*. All were badly infected, showing 100 per cent. fructification after 4 days.

Pods heated at 45°C for 2 hr and immediately inoculated with *S. fructicola* also showed a complete loss of resistance with 100 per cent. fructification.

Pods heated at 44°C for 2 hr and immediately inoculated showed a more or less completely susceptible reaction whether inoculated with *S. fructicola* or *B. cinerea* (except in the case of aged pods where the breakdown was less complete, and the clinical observations showed a susceptible reaction in only two-thirds of the pods); necrotic lesions only were seen in the unheated controls (Table 1; Plate 1, Fig. 1).

When these pods which had been heated at 44°C for 2 hr were stored at 20°C for 3 days before inoculating, a recovery of resistance took place; only necrotic lesions were then seen as a result of inoculation with either fungus, a reaction exactly similar to that of the unheated pods except that the brown pigmentation of the necrotic lesion appeared to be darker in the recovered pods. This recovery was sustained for at least 10 days; then the normal gradual decline in resistance took place due to approaching death of the tissues (Table 1; Plate 1, Fig. 3). However, when the treated pods were stored at 2°C no such significant recovery took place: there was a slight decrease in susceptibility on the second and third days, thereafter all tests showed increasing infection (Table 1).

The pods which were treated with a temperature of 45°C for 2 hr and stored for 3 days at 20°C showed only a slight decrease in the severity of infection after 1, 2, and 3 days. This was not significant enough to constitute a recovery (Table 1).

TABLE 1

BEAN PODS TREATED AT HIGH TEMPERATURES BEFORE INOCULATION WITH S. FRUCTICOLA AND STORAGE

| Treatment | Time of Inoculation after Heating (days) | Total No. of Cavities | No. of Cavities Showing: | | | Clinical Response Rating* |
|---|---|---|---|---|---|---|
| | | | Necrosis | Fructification | Intermediate | |
| Preheating at 44°C for 2 hr, storage at 20°C | Control† | 114 | 114 | — | — | 0 |
| | Immediate | 53 | — | 49 | 4 | 0·96 |
| | 2 | 46 | 37 | — | 9 | 0·095 |
| | 5 | 55 | 49 | — | 6 | 0·05 |
| | 11 | 72 | 49 | — | 23 | 0·15 |
| | Control | 65 | 65 | — | — | 0 |
| | Immediate | 106 | 5 | 101 | — | 0·95 |
| | 3 | 74 | 73 | — | 1 | 0 |
| | Control | 69 | 69 | — | — | 0 |
| | Immediate | 68 | 16 | 38 | 14 | 0·66 |
| | 3 | 77 | 77 | — | — | 0 |
| Inoculated with Botrytis cinerea | Control | 65 | 65 | — | — | 0 |
| | Immediate | 60 | — | 60 | — | 1·0 |
| | 3 | 76 | 74 | — | 2 | 0 |
| Preheating at 44°C for 2 hr, storage at 2°C | Control | 69 | 69 | — | — | 0 |
| | Immediate | 68 | 16 | 38 | 14 | 0·66 |
| | 3 | 68 | 29 | 17 | 22 | 0·41 |
| | 6 | 46 | 10 | 29 | 7 | 0·70 |
| | 9 | 45 | 5 | 33 | 7 | 0·81 |
| | 13 | 43 | — | 37 | 6 | 0·93 |
| Preheating at 45°C for 2 hr, storage at 20°C | Control | 30 | 30 | — | — | 0 |
| | Immediate | 40 | — | 40 | — | 1·0 |
| | 1 | 36 | 2 | 34 | — | 0·94 |
| | 2 | 42 | 4 | 38 | — | 0·90 |
| | 3 | 35 | 4 | 31 | — | 0·88 |

*Numerical assessment as follows: necrosis = 0, fructification = 1·0, intermediate = 0·5.

†In all cases controls were unheated.

Heating at 44°C for 2 hr appears to be a critical time–temperature relation for beans grown at Canberra, so far as resistance to *Sclerotinia* or *Botrytis* is concerned.

(ii) *Low Temperatures*.—Pods stored at −10°C for 24 hr showed complete susceptibility to *S. fructicola*. The tissues were obviously killed and no dynamic resistance was possible.

Table 2 shows the results of inoculating pods kept at 0 and 2°C for various periods; resistance was sustained for about 7–10 days in each case, thereafter they became increasingly susceptible. Pods which were stored at 20°C without pre-treatment had a somewhat longer resistant period, effective change to gradual susceptibility occurring after 13 days (Table 2). However, the colour and external appearance of these pods deteriorated from the eighth day.

TABLE 2

PODS STORED AT LOW TEMPERATURES WITHOUT PRETREATMENT PRIOR TO INOCULATION WITH S. FRUCTICOLA

| Storage Temperature (°C) | No. of Days Stored | Total No. of Cavities | No. of Cavities Showing: | | | Clinical Response Rating* |
|---|---|---|---|---|---|---|
| | | | Necrosis | Fructification | Intermediate | |
| 0 | 4 | 82 | 82 | — | — | 0 |
| | 7 | 69 | 68 | — | 1 | 0 |
| | 13 | 60 | 56 | 2 | 2 | 0·05 |
| | 16 | 58 | 33 | 19 | 6 | 0·38 |
| | 20 | 67 | 20 | 21 | 26 | 0·5 |
| 2 | 3 | 52 | 52 | — | — | 0 |
| | 6 | 49 | 49 | — | — | 0 |
| | 13 | 40 | 30 | 10 | — | 0·25 |
| | 16 | 36 | 20 | 16 | — | 0·44 |
| | 22 | 33 | 5 | 24 | — | 0·79 |
| 20 | 3 | 75 | 75 | — | — | 0 |
| | 8 | 68 | 65 | — | 3 | 0·02 |
| | 13 | 69 | 63 | — | 6 | 0·04 |
| | 16 | 57 | 36 | — | 21 | 0·18 |

*Numerical assessment as in Table 1.

### (b) Influence of Temperature on PA Output

(i) *High Temperatures*.—The pods treated at 45°C were apparently too badly injured by the heat treatment to yield any recoverable PA. Cell sap had exuded into the inoculum drops causing them to spread extensively so that they could not be recovered.

Treated at 44°C for 2 hr and inoculated immediately with *S. fructicola*, the pods yielded PA which varied from 6 to 15 per cent. of that of the control, indicating that defence was slight (see Table 1; Plate 1, Fig. 2). After storage of pods at 20°C for 3 days before inoculation the PA recovered showed again a high activity, invari-ably 10 units, whereas the control varied between 6 and 9 units. The PA activity

started to decrease between the sixth and tenth day. Graphical representation of a bioassay is shown in Figure 1.

On the other hand, when similarly treated pods were stored at 2°C there was only slight production of the active principle. The rating was slightly, but not significantly, increased between the first and third day and progressively decreased thereafter. The results, both as regards immediate and delayed inoculation, were essentially the same whether inoculation was with *S. fructicola* or *B. cinerea*.
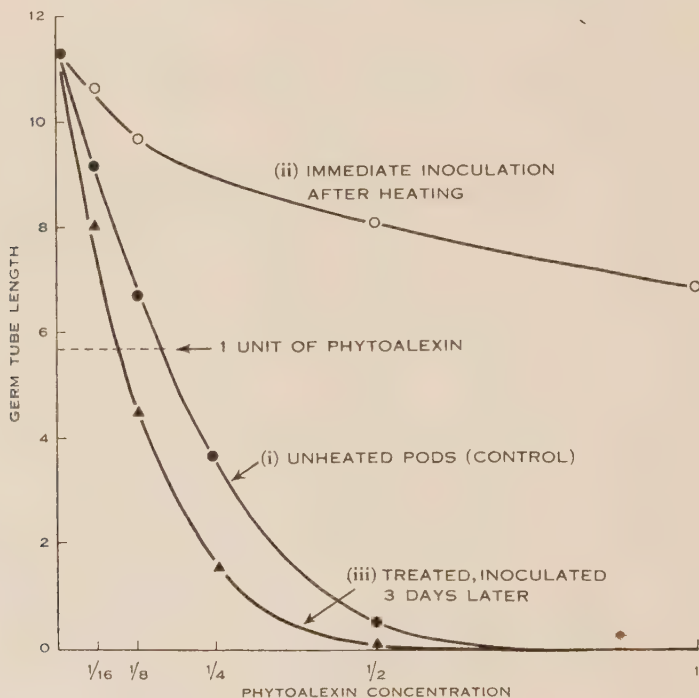


Fig. 1.—Bioassay of phytoalexin (i) from unheated pods (control), (ii) from pods inoculated immediately after heating at 44°C for 2 hr, and (iii) from recovered pods after heating and storage at 20°C for 3 days.

(ii) *Low Temperatures.*—No PA could be collected from pods treated at −10°C, due to disintegration and spread of the drops. In pods stored at 0°C, the PA activity remained high, only slightly less than the control, as did that from pods stored at 2°C until the fourth to sixth day; thereafter a gradual and steady decline in activity took place.

Where pods were stored at 20°C the activity rating of PA was high for 13 days, and only after that time was a decline in activity recorded. This temperature obviously causes less disturbance of the PA-producing mechanism than any of the others used in these experiments.

(c) *Correlation between Clinical Behaviour of the Pods after the Various Treatments and the Activity of PA Recovered from these Pods*

Tables 1 and 2 give the clinical rating under the various treatments but do not show the relevant PA output. Figure 2 shows the relationship between these two phenomena for the experiments in Tables 1 and 2, as well as for additional treatment
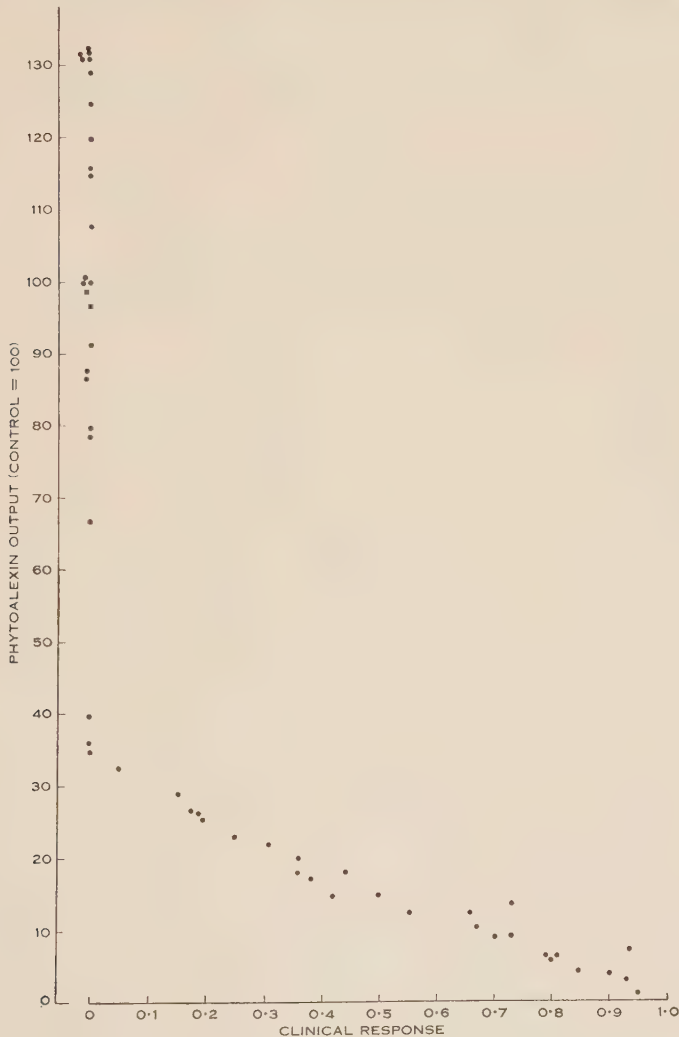


Fig. 2.—Relation between phytoalexin output and clinical response under all variations of treatments.

not cited. There can be no doubt that these two phenomena—PA production and resistance to infection—are very closely linked. It is interesting to note here that the concentration of PA at which only necrotic lesions occur on the pods, appears to be around 2·5–3 units, according to these results. *In vitro*, 5·5–6 PA units are required to inhibit the pathogen completely. This anomalous situation can be explained by

considering that the local concentration at the tip of a penetrating germ tube is actually very high, and that which diffuses into the drop and is re-collected is much diluted. Müller (1958a, 1958b) has discussed this question in the light of experimental evidence relating to these concentrations.
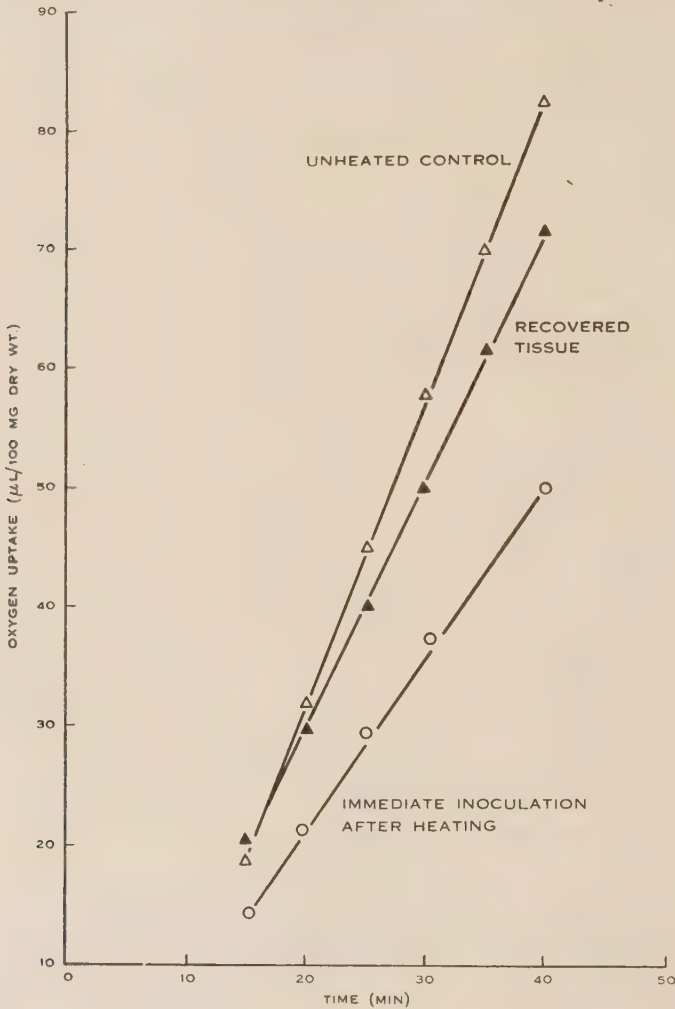


Fig. 3.—Oxygen uptake of unheated tissue (control), of tissue inoculated immediately after heating for 2 hr at 44°C, and of recovered tissue (2 hr at 44°C, 3 days at 20°C).

(d) *Effect of Preheating and Recovery on the Respiration of Bean Pods as Shown by Oxygen Uptake in Air and its Relation to the Clinical Behaviour and PA Output of the Pods after Inoculation with* S. fructicola

(i) *Oxygen Uptake of the Pods.*—The oxygen uptake in air of unheated control pods, of pods heated at 44°C for 2 hr and measured immediately, and of recovered pods which had been stored for 3 days at 20°C after heating, is graphi-

cally represented in Figure 3. The oxygen uptake in pods immediately after heating was only about 60 per cent. of that of the unheated control, the decrease being significant. The recovered tissue, on the other hand, showed a recovery of respiratory activity also, and $Q_{O_2}$ of this tissue was usually around 90 per cent. of control but was not significantly different from control in some experiments (Table 3). An exception was seen in experiment 5 (Table 3) where aged bean pods were used. $Q_{O_2}$ was low in unheated pods, and after heating it was found that uptake had only decreased by 15 per cent. (as compared with 40 per cent. decrease in younger pods), the recovered tissue here showed an increase over control uptake by as much as 15–30 per cent.

TABLE 3

RELATION BETWEEN OXYGEN UPTAKE, PHYTOALEXIN PRODUCTION, AND CLINICAL RESPONSE OF PODS AFTER INOCULATION WITH S. FRUCTICOLA

Values are for unheated control pods, for pods heated at 44°C for 2 hr and measured immediately, and for recovered pods which had been stored at 20°C for 3 days. The difference between treatments in experiments 1–4 is significant at the 0·001 level

| Experiment No. | $Q_{O_2}$ ($\mu$l $O_2$/100 mg dry wt./hr) | | | Phytoalexin Output (units) | | | Clinical Response Rating* | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control (a) | Immediate (b) | Recovered (c) | Control (a) | Immediate (b) | Recovered (c) | Control (a) | Immediate (b) | Recovered (c) |
| 1 | 126·0 | 76·0 | 117·0 | 5·7 | 0·6 | 10 | 0 | 0·73 | 0 |
| 2 | 154·0 | 93·0 | 139·0 | 6·6 | 0·5 | 10 | 0 | 0·8 | 0 |
| 3 | 153·0 | 92·3 | 138·0 | 5·0 | 0·5 | 10 | 0 | 0·74 | 0 |
| 4 | 167·0 | 100·8 | 153·0 | 6·0 | 0·3 | 10 | 0 | 0·9 | 0 |
| 5† | 120·0 | 102·0 | 130·0 | 6·6 | 0·9 | 10 | 0 | 0·66 | 0 |

*Numerical assessment as in Table 1.     †Aged beans used for this experiment.

(ii) *Relation between Oxygen Uptake, Clinical Behaviour, and PA Output.*— Pods were either (a) not heated (controls), (b) heated at 44°C for 2 hr and immediately measured and inoculated, or (c) heated at 44°C for 2 hr, then stored at 20°C for 3 days before measurement and inoculation. *S. fructicola* was used in all cases. There appears to be a general relationship between these three phenomena (see Table 3; Fig. 4). The heated tissue, immediately used, had a low $Q_{O_2}$, low PA output, and low resistance. The recovered tissue had high PA output and high resistance but $Q_{O_2}$ was usually significantly lower than the control. (Sometimes, no significant difference appeared between recovered tissue and $Q_{O_2}$.) The respiratory rate therefore, although it fluctuates in a manner similar to the defence reaction, does not appear to be a direct indicator of the potential efficiency of this system.

IV. DISCUSSION

The variable nature of resistance in certain host–parasite combinations has been noted by many workers. Gäumann (1948) discriminates very clearly between plants which resist the attack of a parasite in a static way, due to some structural

barrier or other obstruction, and truly resistant plants. True resistance is seen in plants which react to parasitic attack by some dynamic defence reaction.

Müller and Börger (1940) have stated that this resistance is conditioned by a genetically controlled reaction potential which only becomes operative after physiological contact has been established between host and pathogen. They concluded from their investigations that environmental and ontogenic factors may modify this reaction potential of the phenotype in such a way that the host or part of it may become susceptible, and that therefore there is no absolute "resistance" as such but only a reaction potential in the plant which allows it to respond to an infection with a defence reaction.
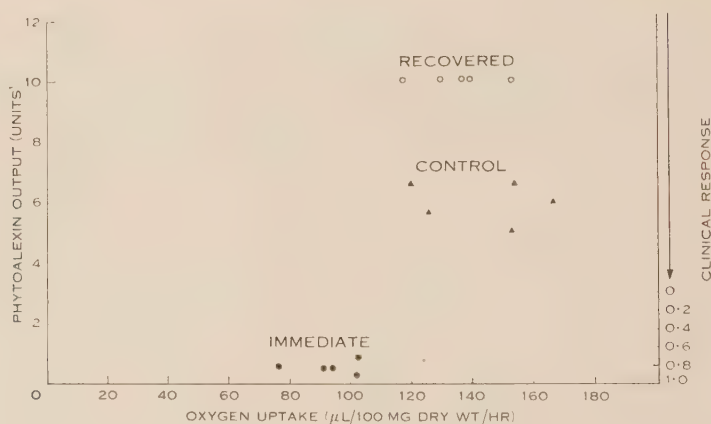


Fig. 4.—Relation between phytoalexin output, oxygen uptake, and clinical response.

Gradinaroff (1943) found that by conditioning potato tubers by preheating he could change their normally resistant reaction to a susceptible one. His clearest results were obtained with treatments at 40 and 45°C for 14 hr. Yarwood (1956) immersed bean leaves (*Phaseolus vulgaris*) in hot water at temperatures of 40, 45, and 50°C for some seconds and increased their susceptibility temporarily towards various viruses and certain fungal parasites. The present investigations yielded results similar to those noted above; for example, bean pods heated at 45°C for 2 hr became and remained completely susceptible to *S. fructicola* whereas unheated pods were resistant to this fungus. This alteration in reaction occurs within certain limits. Temperatures below 45°C do not produce the same effect. Pods heated at 40°C for 18 hr (Müller, unpublished data) showed no breakdown of resistance, while samples heated at 44°C for 2 hr did become susceptible but only temporarily so. At this time–temperature combination, therefore, the breaking of resistance is a reversible process, and this must be considered to be the critical one for our material grown in Canberra. Above 44°C the process is not reversible. So far as low temperatures are concerned pods could be kept for about 7 days at 0 and 2°C in a fully resistant state, whereas at −10°C resistance was completely broken and tissue killed in less than 24 hr.

In Yarwood's (1956) heat-treatment experiments, where the heating was of very short duration, he observed only very temporary increases in number of lesions in some cases; in one case with tobacco mosaic virus, the increased susceptibility lasted at least 3 days. Critical temperatures or times were not studied and indeed the immersion technique produced injury in some cases which nullified the results. In the present investigations, recovery of the ability to resist was found in material treated at 44°C for 2 hr, when the pretreated pods were stored at 20°C. When the storage temperature was 2°C there was no recovery and increasing susceptibility was maintained. It must be concluded therefore that the recovery of resistance in these preheated pods is also controlled by the temperature environment.

Müller (1956) defined phytoalexins as antibiotics which are produced as a result of the interaction of two metabolic systems, host and parasite, and which inhibit the growth of microorganisms pathogenic to plants. They are produced during the first few hours after penetration by the fungus and are dependent on the physiological state of the host cells. When this state is interfered with in any way as for example by treatment with high or low temperatures, the PA production is blocked or modified and resistance is altered accordingly. The variations in resistance seen in the experiments reported here were reflected in the PA production. Solutions of very low potency were produced by treated material which later showed itself to be clinically susceptible both to *S. fructicola* and to *B. cinerea*; whereas recovered or untreated pods, resistant in their reaction to the parasites, normally produced solutions of high activity. These two phenomena are very closely related. In fact, the correlation seen in Figure 2 between clinical behaviour of the pods and PA output was good no matter what treatment had been applied nor whether recovered or unheated pods were used. This reinforces Müller's (1958b) conclusions that blocking of the PA system results in loss of resistance. There seems no doubt that PA is responsible for the death of the pathogen in the resistant host. This is a suprasensitive reaction and the rapidity of the reaction is of the first importance. Plate 2, Figure 4, shows a whole pod in the process of recovering its resistance after preheating. The infection here is confined to anthracnose-like spots; in this case the production of PA has been retarded, thus allowing the fungus to become established on the host tissue. However, as the retardation is only temporary the fungus is soon confined to the single infection spots and spread is arrested. Plate 2, Figure 7, shows a pod partly susceptible and partly resistant, the upper half of it having been treated at a temperature which caused a breakdown of resistance while the other half being held at a lower temperature was not so disturbed.

At the point of penetration in a completely resistant host the pathogen and the host cell are both killed. The active principle recovered from these sites is lethal or inhibitory depending on concentration. It is obvious from these results that there is a threshold concentration of PA necessary for total inhibition of the pathogen. This threshold concentration appears to differ between *in vivo* infections and the bioassays conducted *in vitro*. This difference, however, is more apparent than real, since the PA collected from inoculation sites will be much diluted as compared with the concentration actually occurring at the tip of the penetrating germ tube (see Müller 1958a, 1958b).

The respiratory mechanism was also affected by the heat treatment, a decrease of PA output, resistance, and oxygen uptake being seen in pods measured immediately after heating, when compared with unheated pods of the same bulk. After 3 days storage at 20°C when, by clinical and PA output standards, they had recovered their resistance, they showed also a recovery of oxygen uptake which, nevertheless, did not quite reach the level of the control (except in aged material where recovery rate exceeded that of the control). It seems, however, that the efficiency of the PA production and of resistance to infection does not depend directly on respiration. The defence potential is more sensitive than respiration to the environmental changes, as reductions of the order of 90–100 per cent. in PA output are produced by pretreatment at 44°C for 2 hr, while the reductions in oxygen uptake are only around 40 per cent. with the same treatment.

All the evidence seems to point to enzymatic action being responsible for the various reactions. Müller and Behr (1949) found that narcotics also could reduce the defence potential in potatoes. Since respiration is also affected, the postulated enzyme appears to be linked to the respiratory system and the dehydrogenase system is very tentatively suggested as being initially affected.

This idea of enzymes being involved in the change of resistance was mooted many years ago by Ward (1902). It is possible that the action is "triggered off" by the metabolites of the fungus coming in contact with a respiratory enzyme thereby causing an alteration of the usual respiratory pathway and simultaneously releasing PA or its precursor. The idea of post-infectional respiration changes being involved in the host–pathogen relationship has received much attention from recent workers, e.g. Allen (1953), Shaw and Samborski (1956, 1957), Farkas and Kiràly (1958). The "respiratory factor" reported by Scott, Millerd, and White (1957) is also reminiscent of PA.

Since the production of this defensive substance decreases so markedly after conditioning of this host by extremes of temperature, it would appear reasonable to suggest that each host has its own optimal range of temperature, where its defensive activity is at its highest efficiency; beyond this optimal range the activity is reduced. This may account for the inability of certain plants to resist infection in some climates, while in other climatic conditions the same parasites are not disease factors.

An interesting point emerges from these investigations concerning the problem of facultative parasites. The strain of *B. cinerea* which was used was originally isolated from an infected commercial bean pod. This fungus is known to be a weak parasite, and, as has been shown, was unable to infect normal healthy unheated pods or even preheated and recovered pods. It follows then that the physiological state of the pod from which it was isolated must have been so reduced that no defensive reaction was possible. This could have been brought about by storage conditions. It has been shown that low-temperature storage of material preconditioned by high temperature environment does not permit of any recovery of the defence reaction potential. Only that material which was given a recuperative period at 20°C was able to produce the defence substance in sufficient concentration to avoid disease. This could well be applied to storage of fruit and vegetables, where the usual

immediate low-temperature storage conditions, while inhibiting the growth of the storage disease fungi to some extent, may be doing more harm by reducing too drastically the healthy reactive defence potential of the fruit.

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## EXPLANATION OF PLATES 1 AND 2

### PLATE 1

Fig. 1.—Unheated pod cavities inoculated with *S. fructicola*. Necrotic lesions only.

Fig. 2.—Pod cavities heated for 2 hr at 44°C. Inoculated immediately after treatment with *S. fructicola*. Profuse infection and sporulation.

Fig. 3.—Pod cavities heated for 2 hr at 44°C then stored for 3 days at 20°C before inoculation with *S. fructicola*. Dark brown necrotic lesions only.

### PLATE 2

Fig. 1.—Unheated whole pod inoculated with *S. fructicola*.

Fig. 2.—Whole pod heated at 44°C for 2 hr, then inoculated immediately with *S. fructicola*.

Fig. 3.—Whole pod heated at 44°C for 2 hr, stored at 20°C for 3 days, then inoculated with *S. fructicola*.

Fig. 4.—Anthracnose-like spots of *S. fructicola* on a recovering pod previously heated at 44°C for 2 hr.

Fig. 5.—One infection spot from Figure 4, magnified.

Fig. 6.—Unheated pod, inoculated with *S. fructicola* (control to pod in Plate 2, Fig. 7).

Fig. 7.—Differential heating—loss of resistance in upper half but not in the lower half of pod which had been maintained at lower temperature.

STUDIES ON PHYTOALEXINS. II
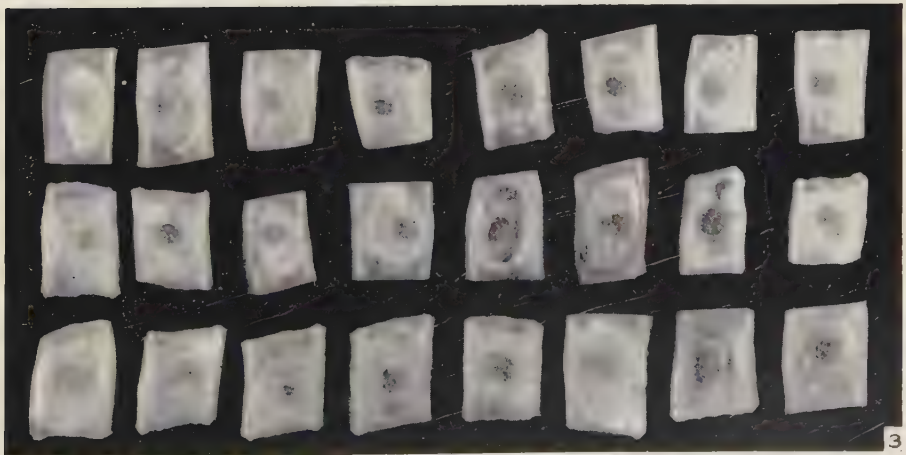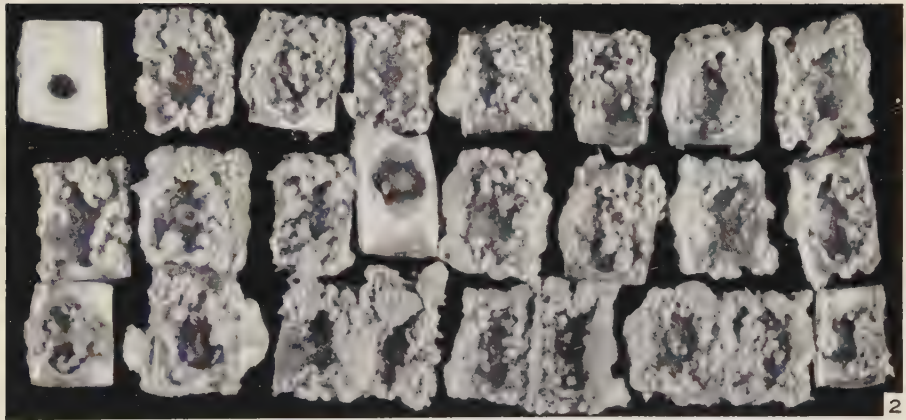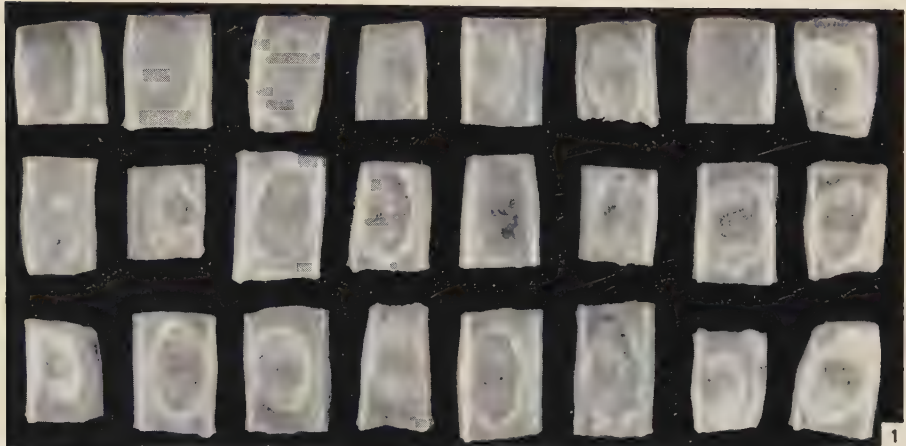
STUDIES ON PHYTOALEXINS. II

# THE PHYSIOLOGY OF SUGAR-CANE

## II. THE RESPIRATION OF HARVESTED SUGAR-CANE

### By R. L. Bieleski*

### Summary

The respiration drift of harvested sugar-cane, variety Pindar, was similar to that of many fruits. Mechanical shock caused the respiration rate to rise over 16–24 hr to a maximum of 80 per cent. above the initial rate; the rate returned to the initial value during the next 72–120 hr. Corresponding changes occurred in the concentration of oxygen in the intercellular atmosphere. At death, only about 30 per cent. of the available carbohydrate had been used. Respiration reached a maximum rate between 37 and 45°C. Above 40°C the respiratory system soon became permanently damaged.

Respiration was not limited by the rate of diffusion of oxygen into the tissues. The concentration of oxygen in the intercellular spaces seldom fell below 8 per cent. at 25°C. The epidermis was not a major barrier to the diffusion of oxygen.

## I. Introduction

Sugar-cane (*Saccharum officinarum*), one of the most productive and photo-synthetically efficient of all crops, has outstanding ability to synthesize and store sucrose, and its physiology merits investigation. Most work already reported on sugar-cane has dealt with practical aspects of its culture (see van Dillewijn 1952), though more fundamental studies have also been made (see Burr *et al.* 1957; Glasziou 1958). The purpose of the present investigations has been to establish the general characteristics of the respiration of harvested stems of sugar-cane.

## II. Materials and Methods

The sugar-cane plant grows in an erect clump of about 8–16 stems. As the internodes mature, the subtended leaves die, so that on a typical 1-year-old plant the leaves grow only at the top of the stout stem, 6–18 ft high. This stem, 10–30 internodes long, cut at the base of the leaves and at ground level, constitutes the "millable" cane used in the following experiments.

The internodes range from 7 to 20 cm long, 2·5 to 4·5 cm diameter, and 60 to 240 g in weight, depending on climatic conditions. Each bears in the old leaf axil a dormant shoot bud and a ring of root primordia, either of which can germinate under suitable conditions. The internode contains several hundred fine vascular bundles, embedded in the parenchymatous storage tissue, and is surrounded by a shell of sclerenchyma and a layer of chlorenchyma one cell thick. The epidermis is small-celled and covered by a thick, waxy cuticle. Primary-stem millable canes,

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9–18 months old, of the variety Pindar, from a commercial crop grown on well-drained alluvial soil at Broadwater, N.S.W., were cut before 10 a.m., and air-freighted to Sydney, being received within 6–48 hr from cutting.

### (a) Effect of Storage Duration and Temperature on Respiration

Both whole canes and single internodes were used. Upon arrival, the canes were trimmed at the node with a saw and the cut ends dipped in a fungicide (0·5 per cent. w/v aqueous "Aretan") and waxed with low-melting-point paraffin. Carbon dioxide output was measured at 25°C by the Pettenkofer method, allowance being made for incomplete absorption at the relatively high rates of air flow used. The time over which the sample was taken depended on the rate of carbon dioxide production, normally being $1\frac{1}{2}$–3 hr for whole canes (about 1000 g) and 4–12 hr for single internodes (about 150–220 g). Temperature effects were determined on single internodes only. After trimming, these were kept at 25°C for 8 days until the respiration plateau was reached. Respiration rates were measured in both ascending and descending steps of temperature. Sufficient time was allowed at each temperature to allow the new equilibrium to be reached.

### (b) Shock Effect

To investigate the effect of shock on respiration, a set of fresh canes prepared in the usual way was allowed to respire for about 250 hr in the respiration chamber. When the respiration rate was steady, the effects of various treatments on the subsequent respiration rate and internal atmosphere composition were noted. Treatments were:

(i) The two terminal internodes were sawn off, the cut ends rewaxed, the cane reweighed and replaced in the respiration chamber.

(ii) Canes were dropped from a known height flat onto a concrete floor covered by a thin layer of cloth, which, with the outer rigid sclerenchyma of the cane, protected the tissues from all but superficial bruising.

(iii) One cane was treated as in (ii) except that it was dropped whilst still sealed inside the respiration chamber.

(iv) Fine sandpaper was used to remove the epidermis from the canes.

### (c) Respiration and Oxygen Supply

The possibility that the respiration rate was limited by the internal oxygen concentration was investigated.

(i) *The Air Space System.*—Transverse, radial longitudinal, and tangential longitudinal stem sections were examined microscopically. Stomatal frequency was studied in epidermis sections, epidermal peels, and epidermis from which all other tissue had been removed by maceration (method of Artschwager 1930).

(ii) *Respiration upon Removal of Epidermis.*—Trimmed and waxed canes were allowed to recover from shock. Then the epidermis and cuticle were removed, using fine sandpaper, and the effect on respiration noted.

(iii) *Respiration and External Oxygen Concentration.*—Canes were prepared as usual, and allowed to recover from shock. After the rates of respiration were established for air, the canes were transferred to 100 per cent. oxygen, 20·5 per cent. oxygen (air), 5·6 per cent. oxygen, and to nitrogen, and the rates again measured.

(iv) *Presence of Alcohol and Organic Acids.*—A cane which had been trimmed, waxed, and stored at 25°C as usual was assayed for both alcohol and volatile organic acids by the method of Fidler (1934). A comparable cane which had been in nitrogen for 2 days was also assayed.

(v) *Composition of the Intercellular Atmosphere.*—Information on the oxygen supply of the internal tissues was obtained from analysis of the intercellular space atmosphere. For sampling the gases, the sample tube method (Wardlaw and Leonard 1939; Trout *et al.* 1942; Smith 1947) was used in preference to the torricellian vacuum method (Magness 1920; Burton and Spragg 1948) which also removes gas dissolved in the tissues. Analysis was carried out in an apparatus based on that of Bonnier and Mangin (Thoday 1913; Wilson 1947). This took a maximum sample volume of 0·25 ml, and was accurate to 0·3 per cent. To obtain the sample the cane was first trimmed and waxed, and a hole 5 mm in diameter was drilled to the centre of the cane at the middle of the internode. The hole was dried out with spills of filter paper. The side of the hole was then lightly seared with a red-hot metal rod to remove attached vascular fragments and immediately waxed over with a thin layer to improve the seal. This treatment damaged only a superficial layer of cells and prevented fungal infection. A tightly fitting capillary tube, drawn out to 3 mm diameter at the outside end, was fully inserted into the hole and waxed firmly into place. A piece of bicycle-valve rubber was fitted on the projecting narrow end, then folded over the tip, and held with copper wire, to give an air-tight valve. A plastic tube of 0·5 mm bore was filled with mercury and connected at one end to the entry tube of the analyser and the other to the valve. When this was opened, a sample of the internal atmosphere could be transferred, with minimum (less than 1 per cent.) contamination from the outside air, directly to the analyser. All experiments were carried out at 25°C.

In one experiment, single internodes were treated in the following ways: unwaxed, waxed at the ends, waxed on the sides but not at the ends, and waxed on both sides and ends. After 24 hr the internal atmospheres were analysed. For the remaining experiments, only the cut ends of the cane were waxed.

To examine the problem of the high resistance in the stem to lateral gas diffusion, internodes were prepared as usual, and after allowing recovery from shock, the internal gas was analysed. Then from one set of internodes, a layer of 1/8 of the cane surface was carefully removed to a depth of about 120–150 $\mu$ (to just below the chlorophyll layer, where the intercellular space system starts). A sharp razor-blade was used, and the tissues were removed very carefully to avoid further shock. From a second set, 1/24 of the surface was removed in the same way, and a third set was kept as control. Gas samples were subsequently analysed.

Gas samples were taken from a region 3 mm from the surface of an internode, and compared with samples taken from the centre of the same internode at the same time, using as sampling tubes hypodermic needles inserted from the waxed end.

The rate of oxygen diffusion into cane and the time required for equilibration were investigated by first freeing the internal atmosphere of oxygen in a slow stream of nitrogen for 24 hr. A cane was then returned to air, and gas samples taken at different times were analysed. Samples were taken from each internode in turn to reduce errors due to entry of outside air. For one representative cane, the fresh weight, average diameter, surface area, and the respiration rate were measured as well as the internal oxygen concentration at final equilibrium.

## III. Results

### (a) Effect of Storage Duration and Temperature on Respiration

The curves of respiration drift shown in Figure 1 were typical of those obtained for single internodes and whole canes. The gross irregularities in carbon dioxide production so often encountered in similar studies on fruits were absent, except in a few samples where fungal infection occurred. The only striking feature of the curve
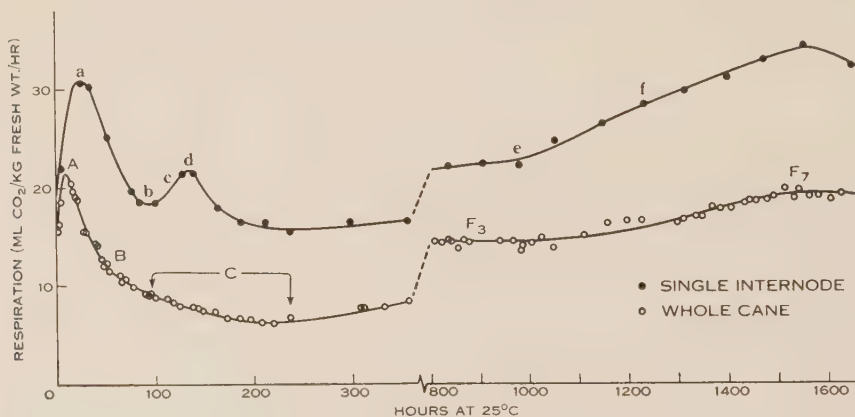


Fig. 1.—Respiration drift of a single internode and a whole stem of harvested sugar-cane. $a$, $A$ (after 16–24 hr), initial peak in respiration; $b$, $B$ (after 90–110 hr), end of initial respiration peak; $c$, $C$, first signs of root germination; $d$ (after 135–155 hr), secondary respiration peak, always 30–50 hr after first signs of root germination, and apparently related to it—often absent in whole canes; $e$ (after 800–1400 hr), senescent respiration rise; $f$, first signs of death, single internode, $F_3$, of internode 3, whole cane, $F_7$, of internode 7, whole cane. Internal cells died first, as shown by a red pigment formation, associated with cell death in sugar-cane.

was the initial sharp rise and fall in respiration rate ($a$, $A$, Fig. 1). This was usually higher for single internodes and for younger internodes, rising to as much as double the starting level. Occurrence of the rise was independent of the length of time from harvesting and appeared related to the trimming and waxing procedure. The possibility of its being a temperature effect was eliminated. At death, about 30 per cent. of the total available carbohydrate had been utilized, the remainder (often largely as hexose, formed through inversion) being left in the dead tissues.

The effects of temperature on respiration are shown in Figure 2. Respiration attained its new level almost as soon as the temperature of the cane reached equilibrium.

### (b) Shock Effect

(i) When the sawing and waxing procedure was repeated on canes which had passed the initial peak, the respiration rate rose sharply over the following 20–24 hr to a peak almost 75 per cent. higher than the initial rate, and then returned slowly over the next 120–170 hr to the initial level. The induced peak was thus identical in shape with the initial peak (Fig. 3). This confirmed the observation that a factor associated with trimming and waxing might be responsible for the initial peak.

(ii) There was a similar rise in respiration of 15 per cent. in canes dropped three times from 1 ft on to a concrete floor, and of 31 per cent. with canes dropped six times from 2 ft. The control canes showed that this was not due to opening the respiration chamber or to removing and replacing the canes. This indicated that mechanical shock was responsible for the effect.
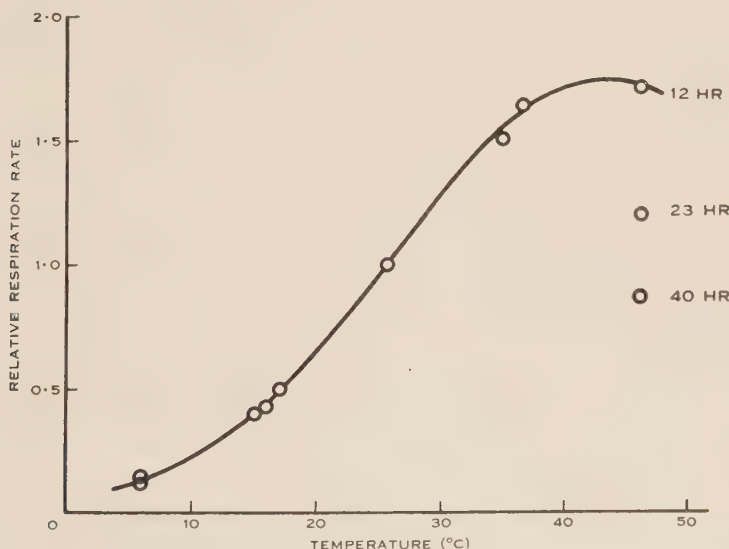


Fig. 2.—Effect of temperature on the respiration rate of single internodes of sugar-cane, relative to the rate of 25°C. Rates measured after 12 hr at the given temperature, except where stated. At 36°C, respiration was constant over 2 days, but, at temperatures above 40°C, fell with time. At 47°C definite signs of damage appeared after 40 hr and the tissue did not recover at 25°C.

(iii) This was confirmed when one cane showed the effect after being given the shock within the chamber itself.

(iv) When the heavily cutinized, waxy epidermis was removed with very fine sandpaper, the effect on the respiration rate (rising 85 per cent. in 26 hr, and falling back to the original level in a further 120–150 hr) was identical to that caused by severe shock.

(v) When the internal atmosphere changes following the trimming were studied, the oxygen concentration, initially 8–12 per cent. fell to 3·5–6 per cent. within 5–15 hr and then returned to 7·5–11·5 per cent. during the following 20–36 hr. There

was a tendency for the minimum oxygen concentration to be lowest and the duration of the depression to be longest with the youngest internodes. The internal carbon dioxide concentration, 9–12 per cent., did not change significantly during the experiment. The changes in oxygen concentration occurred more rapidly than those in carbon dioxide output. The explanation for this difference ·as well as for the stability of the internal carbon dioxide levels, can probably be found in a "buffering" effect due to the relatively high solubility of carbon dioxide in the cell fluids.

### (c) *Respiration and Oxygen Supply*

(i) *The Air Space System.*—The tissues were dense, the intercellular space ranging from 1 to 6 per cent. and averaging 3 per cent. (4–7 ml gas/internode). The intercellular space formed a regular, well-connected system, interrupted only at the nodes, running along the length of the cane; but across the cane it was poorly developed and discontinuous. Rough measurements on the rate of air flow through longitudinal and transverse cores of the storage tissue confirmed this difference.
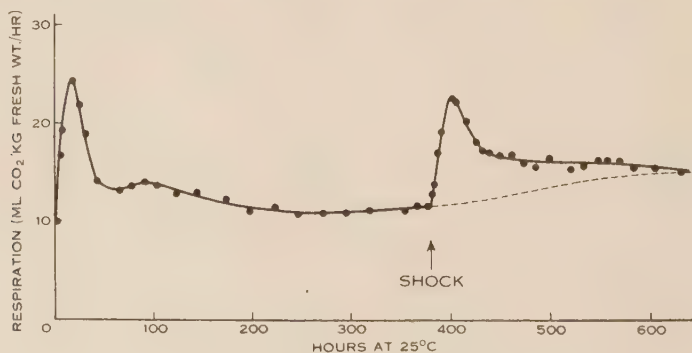


Fig. 3.—Effect of severe shock on the respiration of sugar-cane. – – – – Control.

The intercellular spaces were much smaller near the outside of the stem. Minute spaces were found at the cell corners out to the inner side of the peripheral vascular bundles (600–1200 $\mu$ from the surface); in the gaps between the bundles they extended as far as the chlorenchyma layer, within 90–120 $\mu$ of the surface.

It was not possible to determine the frequency of the stomata on the stem, as it was only occasionally possible to identify a stoma positively even in epidermis from young internodes. This was due to the epidermal cells being very small, with thick, highly convoluted cell walls, and to the presence of so-called "silica cells" which were identical in appearance to the accessory cells. For example, the epidermal cells, although 80–100 $\mu$ long, were only 10–20 $\mu$ wide, with the thick walls making up a third of the width. The accessory cells were relatively thin-walled, and 10–12 $\mu$ long by 12–15 $\mu$ wide, as were the silica cells; while the entire guard cell pair plus the stoma was only 6–8 $\mu$ wide by 10–15 $\mu$ long. It did, however, appear that the stomata were infrequent as well as very small (cf. Artschwager 1930; van Dillewijn 1952).

(ii) *Respiration upon Removal of the Epidermis.*—The results of this experiment are quoted in Section III(*b*)(iv). The respiration rate rose markedly after treatment, but returned to the initial level after 5–6 days. The rate of water loss from similarly treated material (seven times higher measured in still dry air) did not alter over a much longer period. The behaviour, which is difficult to explain by increased respiration due to a reduced diffusion resistance, corresponds exactly to a severe shock effect.

(iii) *Respiration and External Oxygen Concentration.*—The respiration rate in 100 per cent. oxygen was only 12 per cent. higher than in air, whereas in 5·6 per cent. oxygen it was 33 per cent. lower, and in nitrogen 60 per cent. lower.

(iv) *Presence of Alcohol and Organic Acids.*—In the cane stored in air, any alcohol present was below the limit of detection (2 mg alcohol/kg fresh wt.), and the amount of volatile organic acid (estimated as acetic acid) was small, averaging 25 mg/kg fresh wt. In the cane stored 48 hr in nitrogen the amount of volatile acid, 35 mg/kg

TABLE 1

EFFECT OF VARIOUS WAXING TREATMENTS ON INTERNAL ATMOSPHERE COMPOSITION OF SINGLE INTERNODES

| Treatment | Carbon Dioxide (%) | Oxygen (%) | Nitrogen (%) |
|---|---|---|---|
| Completely unwaxed | 0·5 | 19·5 | 80·0 |
| Cut ends only waxed | 10·5 | 9·0 | 80·5 |
| Stem surface only waxed | 1·0 | 18·0 | 81·0 |
| Completely waxed | 29·0 | 0·5 | 70·5 |
| External atmosphere | 0·0 | 20·5 | 79·5 |

fresh wt., was not significantly greater though the alcohol content was 310 mg/kg fresh wt. An aldehyde test was negative. The calculated rate of alcohol formation corresponds closely with the known rate of anaerobic carbon dioxide production in sugar-cane (0·14 m-equiv. alcohol/kg fresh wt./hr, and 0·17–0·27 m-equiv. $CO_2$/kg fresh wt./hr). It appears therefore than in cane in air at 25°C there is no anaerobiosis.

(v) *Composition of the Intercellular Atmosphere.*—The effects of different waxing methods on the composition of the intercellular atmosphere are shown in Table 1. They show that there can be little resistance to gas diffusion along the length of the cane. This explains why in the whole cane there is little variation in gas composition along the length, despite the higher respiration rates in the younger internodes (see below). On the other hand, the resistance to lateral diffusion is quite high. Results for the completely waxed internodes show that the waxing and sampling techniques prevented any serious contamination of the sample by the outside air. The apparent lowering of the nitrogen concentration was undoubtedly due to anaerobic carbon dioxide produced either increasing the internal atmosphere pressure or displacing the nitrogen.

In end-waxed canes, the oxygen concentration was generally higher in the whole canes than in single internodes. For any one cane, the level was fairly constant from internode to internode, though varying from time to time—the values being carbon dioxide 5·5–9·0, oxygen 11·0–14·5, and nitrogen 79·0–81·0 per cent. respectively. With single internodes, there were greater variations both with respect to time and from one internode to another. Levels were: carbon dioxide 8·0–13·0, oxygen 3·5–11·5, and nitrogen 79·0–87·0 per cent. The changes followed a definite pattern, due largely to the "shock effect" (see Section III(*b*)(v)). A stable level was not reached until 2–3 days after the preparation of the internodes for internal atmosphere sampling.

TABLE 2

EFFECT OF INTERNODE AGE ON INTERNAL ATMOSPHERE COMPOSITION OF SINGLE INTERNODES OF ONE CANE

| Gas* (%) | Internode Number† | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Carbon dioxide | 12·8 | 11·2 | 11·2 | 11·2 | 10·3 | 10·7 | 10·2 | 10·4 | 9·1 |
| Oxygen | 7·1 | 6·7 | 6·8 | 7·5 | 8·1 | 9·5 | 11·0 | 10·0 | 12·2 |
| Nitrogen | 80·1 | 82·1 | 82·0 | 81·3 | 81·6 | 79·8 | 78·8 | 79·6 | 78·7 |

*Values taken 48 hr after trimming.

†Internode numbers were defined such that internode 1 was the youngest mature internode, internode 2, the second-youngest mature one, etc.

When the various gas concentrations were stabilized, there were still differences between internodes, related to their position on the stem, and thus to their age. Levels ranged from carbon dioxide 8·0–10·0, oxygen 10·0–12·0, and nitrogen 79·0–81·0 per cent. for the oldest basal internodes to carbon dioxide 11·0–13·0, oxygen 6·5–8·0, and nitrogen 80·5–82·0 per cent. for the second-youngest mature internodes. The differences are not great, probably since the internodes were all mature despite their great differences in age (approx. 9 months and 6 weeks respectively) (Table 2).

Canes with part of the epidermis removed showed no changes in the internal atmosphere. If the outermost 150 $\mu$ of tissue formed a major barrier to diffusion, the treatment should have increased the internal oxygen supply. The resistance of the epidermis to gas diffusion must, therefore, be relatively low.

Analyses of gas samples taken near the epidermis are shown in Table 3. If the cuticle and epidermis had formed a major barrier to gas diffusion into the cane, the rate of oxygen diffusion through the outermost 3 mm would have been considerably lower than that through the rest of the tissue. On the other hand, if there were no epidermal barrier the two rates would be expected to be approximately the same.

and (modifying the equation for gas diffusion into a uniform cylinder (Fenn 1927), cf. Section IV)

$$\frac{C_o - C_r}{C_o - C_a} = \frac{a^2 - r^2}{a^2},$$

where

$C_o$ = oxygen concentration outside tissue,
$C_r$ = oxygen concentration $r$ cm from centre of cylinder,
$C_a$ = oxygen concentration at centre, and
$a$ = radius of cylinder,

will hold. Here, this relationship is satisfied, so the rate of oxygen diffusion through the outermost 3 mm is approximately equal to the rate through the rest of the tissue. The rate of oxygen diffusion into oxygen-free cane is shown in Figure 4.

TABLE 3

EFFECT OF THE PERIPHERAL TISSUES OF THE INTERNODE ON THE INTERNAL ATMOSPHERE COMPOSITION

| Site of Sample | Carbon Dioxide (%) | Oxygen (%) | Nitrogen (%) |
|---|---|---|---|
| Outside air | 0·0 | 20·3 | 79·7 |
| 3 mm from cane surface | 4·2 | 16·3 | 79·5. |
| 16 mm from cane surface, at centre | 10·5 | 9·3 | 80·2 |

The approximate equilibrium was reached in about 70 min. This rapid attainment of equilibrium implies a fairly ready diffusion of oxygen into the cane tissues. It also shows that, in general, the minimum time allowed for equilibration, 6 hr, was fully adequate.

For the cane above (Fig. 4), the following measurements were also made:

| | |
|---|---|
| Surface area of cane | 1700 sq. cm |
| Fresh weight of cane | 1·44 kg |
| Average diameter | 1·7 cm |
| Internal oxygen concentration at equilibrium | 0·135 atm |
| External oxygen concentration | 0·205 atm |
| Respiration rate of cane | 15·6 ml $CO_2$/hr |

IV. Discussion

(a) *Effect of Storage Duration and Temperature on Respiration*

Sugar-cane, despite its morphological status as a stem, has many characteristics of a fruit (e.g. high sugar content), and most of the features of the respiration drift are typical of fruits. The respiration rates compare well with those quoted

by other workers for other varieties (Kamerling 1904; Khanna and Raheja 1938, 1948a, 1948b; Hes 1949). Those of Lal and Srivastava (1945) were considerably higher, but there is a strong possibility that their measurements, made approximately 18 hr after trimming, were made while the shock respiration was at a peak.

The rate for mature stems of Pindar variety was found to be in the range 9–18 ml $CO_2$/kg fresh wt./hr $(Q_{CO_2} = 0.032–0.064 \, \mu l \, CO_2$/mg dry wt./hr). This rate of respiration is comparable with that of the slower-respiring fleshy storage organs (cf. apple fruits and potato tubers, 10–25 ml $CO_2$/kg fresh wt./hr). This low level in cane is doubtless due in part to the high proportion of non-living organic matter, since sugars form 40 per cent. of the dry weight, and fibre, mainly cellulose, another
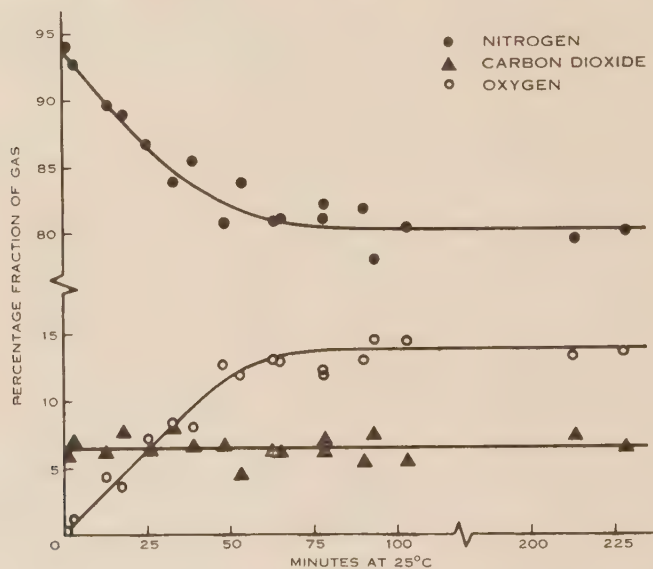


Fig. 4.—Diffusion of oxygen into an initially oxygen-free stem of sugar-cane.

30 per cent. Respiration, however, can be considerably higher, as in the initial peak; it appears in some way to be controlled at a low level. It is unlikely that the amount of respirable material is limiting, unless the permeability of the tonoplast is so low that sufficient sugar cannot move to the cytoplasm.

Death of the cells occurred long before all the sugar was utilized, the sugar lost accounting exactly for the carbon dioxide formed if the R.Q. is assumed to be 1. These are common features of the respiratory drift of fruits and storage tissues (e.g. apple, Kidd and West 1930; Onslow, Kidd, and West 1931; potato, Barker 1936).

The dual effect of temperature on respiration is a general phenomenon in plants (James 1953), though the temperature at which the secondary depression commences is higher for sugar-cane (40°C) than the usual range (25–35°C). Despite this, such temperatures may be met in the field. Khanna and Raheja (1938) found a critical temperature of 39–41°C for respiration of sugar-cane leaves in a field experiment.

### (b) Shock Effect

The initial peak in respiration rate is not a wound effect in the usual sense, for even where the only tissue cut has been the two trimmed ends of the cane, the respiration has risen over 50 per cent. Such wounding could account only for a rise of 0·5 per cent. in the respiration of the whole cane. Observations on internal atmospheres show that the effect is not due to increased access of oxygen to the tissues. If there is any physical change causing the respiration rise, it must operate at the cellular or subcellular level.

The phenomenon is strikingly similar to the "handling respiration" obtained by Audus (1935, 1940) with cherry-laurel leaves, and Barker (1935) with senescent potatoes. With sugar-cane, the respiration rate roughly doubled in 16–24 hr, returning to normal over the next 120 hr; with cherry-laurel leaves, the maximum was reached in 2–6 hr, returning to normal over the next 60 hr; with potato, the maximum (only 30 per cent. higher) occurred in 18 hr returning to normal over the next 200 hr. The internal changes of oxygen concentration in sugar-cane correspond more closely on the time scale than do the external changes of carbon dioxide concentration to the cherry-laurel results. As with the cherry-laurel leaves, there was some indication that the effect was less pronounced in more senescent canes; the effect was also less pronounced in the older tissues.

The term "shock respiration" rather than "handling respiration" is used here to distinguish the type of stimulation required to evoke the effect. Handling is all that is required with flexible leaves and softened potatoes, but a shock (e.g. vibration) is necessary with the rigid, sclerenchyma-protected sugar-cane. It is believed, however, that except for differences caused by the different physical structures of the experimental materials, the two effects are identical. This illustrates that even with rigid tissues, manipulation and handling can affect the respiration markedly, and may introduce an uncontrolled factor in experiments.

Sugar losses through shock are of little commercial importance even though the effect must be induced by harvesting procedure. The maximum loss would be only 0·5 per cent. of the available sugar.

### (c) Respiration and Oxygen Supply

The observations showed that no barrier existed in the storage tissue, where gas diffusion was rapid and uniform and air spaces were frequent. However, no air spaces could be seen in the outermost $100-600\,\mu$ of the stem and stomata were infrequent; and so it was considered whether the epidermis or the narrow outer layer of the stem might form a major barrier to gas diffusion, as in the apple (Trout et al. 1942) and potato (Burton 1950). The results clearly demonstrate that there is no such barrier, and that the rate of oxygen diffusion through the tissue is adequate to support respiration at 25°C. The measurements of respiration in 100 per cent. oxygen confirm this observation. However, the increase of respiration rate with temperature is such that above 35°C, anaerobiosis must at times occur, even in air, in the innermost tissues of the internode.

The respiration rate at 25°C is almost independent of the external oxygen concentration over the range 15–100 per cent. oxygen, corresponding to an internal oxygen concentration of 5–90 per cent. Analysis of the internal atmosphere of sugar-cane internodes have previously been made by Bonazzi (1931), who obtained very large differences between young and old internodes. In the present work only slight differences were found. Bonazzi used the unsatisfactory torricellian vacuum technique which extracts dissolved gases, invariably high in carbon dioxide. Analyses which are uncomplicated by any dissolved gases give a better picture of the internal changes.

From the data, calculations of an "invasion coefficient" for oxygen movement into sugar-cane tissues can be made, considering sugar-cane as an infinitely long cylinder. Fenn (1927) gives the equation for a cylinder of uniformly respiring material:

$$C = C_o - A(a^2 - r^2)/4D,$$

where

$C$ = concentration of oxygen (atm) $r$ cm from the centre of the cylinder,
$C_o$ = concentration of oxygen at the surface of the cylinder ($a = r$),
$a$ = radius of the cylinder,
$A$ = rate of oxygen consumption, in ml/ml tissue/min, and
$D$ = diffusion constant of oxygen in ml/sq. cm/min with gradient 1 atm/cm.

($D$ here is better called the invasion coefficient, since the measured value includes diffusion both through the air in intercellular spaces and through the water in cells).

This can be rearranged in terms of the invasion coefficient,

$$D = \frac{Aa^2}{4(C_o - C_a)},$$

where $C_a$ = concentration of oxygen at the centre ($r = 0$).

It can be assumed that the respiration rate in sugar-cane is uniform throughout the tissues; experiments have indicated that there is no pronounced gas diffusion barrier at the epidermis, and that the rate of lateral gas diffusion is fairly uniform. Under conditions where $C_o$ is uniform along the cylinder, the longitudinal gas diffusion in the stem can be neglected and the above equation applied. Here,

$A = 1.8 \times 10^{-4}$ ml $O_2$/ml/min, assuming R.Q.$=1.0$ and density of cane$=1.05$,
$a = 1.7$ cm,
$C_o = 0.205$ atm,
$C_a = 0.135$ atm, and thus
$D = 1.9 \times 10^{-3}$ ml/sq. cm/min for gradient 1 atm/cm.

This value of $D$ has been obtained from measurements (within the normal range) on only one cane.

The value of $D$ for oxygen diffusion through water is $3.4 \times 10^{-5}$ ml/sq. cm/min (Krogh 1919) so the rate of oxygen diffusion through the storage tissue of sugar-cane is some 60 times as high as through water. This is in line with a similar observation made for the potato (Burton 1950), and as in that case, it appears that this

great increase in the diffusion rate is brought about by the presence of very small air spaces at the cell corners. In air, the rate of oxygen diffusion at 25°C is 12·5 ml/sq. cm/min, which suggests that in sugar-cane the approximate effective cross-sectional area of the lateral air space system is 0·015 per cent. of the total area. Oxygen diffusion laterally through sugar-cane ($D = 1·9 \times 10^{-3}$ ml/sq.cm/min) is thus almost 10 times slower than through the storage tissues of the potato ($D = 1·74 \times 10^{-2}$ ml./sq.cm/min and equivalent air space cross section $= 0·15$ per cent. (Burton 1950)); even though, the percentage air space in sugar-cane (3 per cent.) is higher than in the potato (1·5 per cent.). In sugar-cane, unlike the potato, there is a marked anisotropy in the intercellular space system. The main air space channels run longitudinally and would not aid lateral diffusion, the lateral system being but poorly developed.

## V. Acknowledgments

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# THE RELATIONSHIP OF HIGH-ENERGY PHOSPHATE CONTENT, PROTEIN SYNTHESIS, AND THE CLIMACTERIC RISE IN THE RESPIRATION OF RIPENING AVOCADO AND TOMATO FRUITS

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*Summary*

The relationships between respiration rates and the content of protein nitrogen and high-energy phosphate were investigated in ripening avocado and tomato fruits. The climacteric rise in respiration rate was accompanied by a rise in total high-energy phosphate in avocados ripened after picking; in tomatoes ripening on the plant the change in total high-energy phosphate was not significant. The ratio of protein nitrogen to total nitrogen increased during the climacteric rise in both fruits. It is concluded that, while the rates of respiration and synthetic processes may be controlled by the phosphate transfer system, the evidence suggests that uncoupling of respiration and phosphorylation does not explain the respiratory climacteric.

## I. Introduction

Recent speculations on the control of respiration rate in ripening fruits have associated the mechanism with the generation of high-energy phosphate ($\sim$P). Millerd, Bonner, and Biale (1953) and Pearson and Robertson (1952, 1954) showed that the respiration of tissue cut from pre-climacteric fruits was markedly stimulated by 2,4-dinitrophenol, but tissue cut from fruit at the climacteric showed little stimulation. In both papers, it was suggested that the concentration of phosphate acceptor, adenosine diphosphate (ADP), limited the rate of respiration of immature fruit and that this limitation was removed as the fruit matured. Millerd *et al.* considered that, as the fruit (avocado) ripened, a compound was formed which uncoupled phosphorylation and respiration, thus preventing re-synthesis of adenosine triphosphate (ATP). With the consequently rising concentration of ADP, the limitation previously imposed on the respiration rate would be removed. Pearson and Robertson (1954) suggested that the increasing demands for the maintenance of protein and other unstable compounds in the cell might reduce the concentration of ATP and increase that of ADP to bring about the same effect on respiration rate. These workers showed that net protein synthesis would, in fact, continue after the rise in respiration. Hulme (1954) showed that the ratio protein/total nitrogen ran

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parallel to the rate of respiration of apple fruit. Marks, Bernlohr, and Varner (1957) have shown that the percentage esterification of $^{32}P$ reached a maximum in pre-climacteric tomato fruit and did not decrease until after the onset of pink colour; 2,4-dinitrophenol applied to green mature fruits inhibited the esterification, and prevented normal ripening.

The objects of the experiments reported here were to measure the concentration of $\sim P$, protein nitrogen/total nitrogen ratios, and respiration rates at different stages of ripeness in avocado and tomato fruits. This work is preliminary to programmes on fruit ripening now being carried out independently at Davis, California, and at Melbourne, Australia.

## II. Materials and Methods

The avocados (var. Anaheim) used in these experiments were obtained from Murwillumbah, N.S.W. Fruits were harvested early in the morning and sent to Sydney by air freight. On the evening of the same day, they were examined at the laboratory and placed in a room at 25°C. Single fruits were placed immediately in the respiration chambers of a Pettenkofer apparatus, through which a current of $CO_2$-free air was passed continuously; measurements of the respiration rates began the next morning. Fruits were sampled immediately and after various intervals of time depending on the probable state of ripeness as deduced from the individual respiration curves and the rate of softening of the fruit. As fruits were taken for analysis, they were replaced in the chambers by other individuals which had been kept at the same temperature. The time of sampling individual fruits was chosen so that the various samples would represent different stages of ripeness and different points on an idealized climacteric curve of respiration. On removal from the respiration chambers, some of the tissue was used for the extraction of acid-soluble phosphates and some was dried for subsequent dry weight and nitrogen determinations. This procedure was followed with two different lots of fruit, providing data from a total of 13 individuals; these data were combined for discussion as one experiment.

Tomatoes (var. King of All) were harvested early in the morning from unheated glass-houses near Sydney and taken to the laboratory. The fruits were sorted into lots of eight different stages of ripeness, judged by external appearance as follows: (1) slightly immature, entirely green with little or no cork formation at the stem scar; (2) mature green; (3) first trace of colour; (4) about half-coloured; (5) orange; (6) light red; (7) full red; (8) soft ripe. Within each colour grade fruits were matched into paired samples of 12 fruits each. One of these samples was placed in a respiration chamber at 25°C, and the other was used for analysis. Respiration measurements were taken by the Pettenkofer method on the next 2 days after harvest, and these readings were averaged to represent the probable respiration rate of the paired sample; the latter was analyzed immediately after harvest for acid-soluble phosphates, dry weight, and nitrogen.

In order to minimize analytical variation due to tissue differences within the fruit, these procedures were followed: Each avocado was cut into 16 approximately

equal radial longitudinal slices, the first cut being made on the axis of bilateral sym-metry. Alternate sixteenths were taken, one for drying and the other for phosphate extraction. With tomatoes, each fruit in the sample was also cut into 16 radial longitudinal slices. Where seeds were found they were removed. Every fourth slice was used for extraction, so that the total extraction sample consisted of four-sixteenths of each of 12 fruits. One-sixteenth of each fruit was taken for the drying sample.

The extraction of the acid-soluble phosphate was carried out at 0°C. The chopped fruit tissue was placed in a Waring Blendor with a measured volume of $1.2N$ $HClO_4$ ($1.5$ ml/g fresh tissue), and blended at full speed for 3 min; the sample was then centrifuged at approximately $1500\,g$ for 15 min, the supernatant was poured off and filtered, and the filtrate was adjusted to pH 7.6 with KOH, the volume of filtrate taken being recorded before and after neutralization. After an interval of frozen storage, the $KClO_4$ precipitate was centrifuged off and the phosphate fractions were subsequently determined on the supernatant. High-energy phosphate was measured by the enzymic method of Slater (1953) as modified by Rowan (1958). Uridine triphosphate (UTP), which is included in the analysis of $\sim$P, is present in both fruit, but chromatographic examination has shown that ATP is the pre-dominant nucleoside triphosphate (Rowan, unpublished data). For nitrogen and dry weight determinations, the tissue was dried in an air-draught oven at 70°C to constant weight (12–16 hr); this was followed by drying in a vacuum oven for 6 hr, and the dried sample so obtained was ground in a Waring Blendor. Nitrogen determin-ations were by the Parnas-Wagner method (Turner 1949); the protein nitrogen was taken as that fraction insoluble in 70 per cent. ethanol.

## III. Results

### (a) Avocados

Drifts in respiration of different individual fruits appeared to follow a similar pattern, the rate decreasing to a pre-climacteric minimum, increasing to a climacteric maximum 24–36 hr later, and then decreasing again slowly. The time required to reach the pre-climacteric minimum varied between individual fruits. In pre-senting the data (Fig. 1(a)) the placing of the curve for each fruit on the time-axis is arbitrary, but is based on the comparative ripeness of the individual fruits at the time of the final sampling and an estimation of the probable time of occurrence of the rise from minimum to maximum. Figure 1(b) shows all estimations of the total $\sim$P concentrations determined in the same fruits when they were taken from the respiration chambers.

These results show that the concentration of $\sim$P tends to follow the drift of respiration rate from the time of the pre-climacteric minimum onwards, with a maximum $\sim$P concentration at the peak of the climacteric. The concentration of protein nitrogen in the fruit was closely correlated with the concentration of $\sim$P. However, the ratio of protein nitrogen to total nitrogen (Fig. 1(c)) rose steadily during ripening suggesting that the levels of $\sim$P were adequate for this synthesis.

### (b)  Tomatoes

The results for the tomatoes are shown in Figure 2.  The respiration rates and analytical values are plotted against the eight stages of ripeness at which the fruits
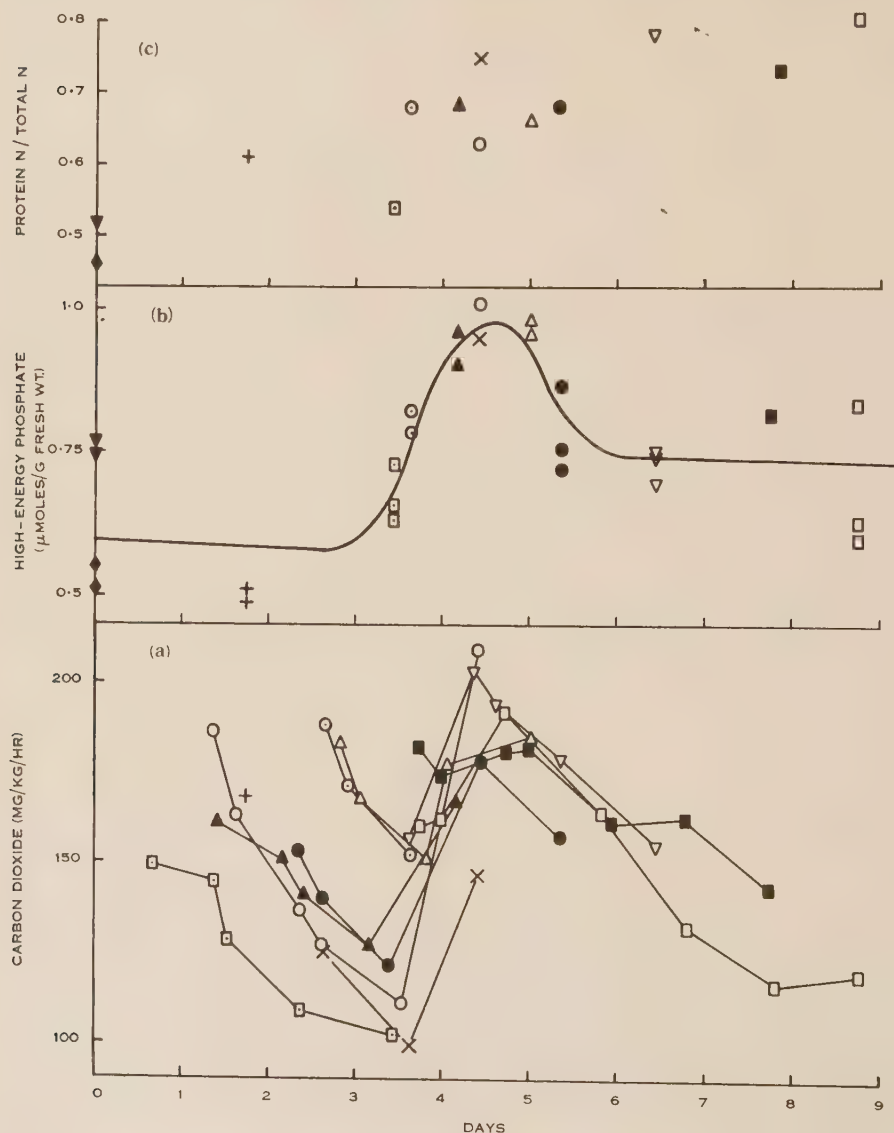


Fig. 1.—Changes in avocado fruit with time.  (a) Rate of respiration per gram fresh weight; (b) concentration of high-energy phosphate per gram fresh weight; (c) ratio of protein nitrogen to total nitrogen.

were sampled.  Using these stages of ripeness as an arbitrary time scale, the respiratory pattern shows the expected climacteric rise (Fig. 2(a)).  It has been demonstrated with tomatoes that such a curve can be established by this means or

by following the respiration rate during ripening through the same colour stages of a single sample of mature green fruits (Beadle 1937; review in Workman, Pratt, and Morris 1957). A rapid rise in respiration is noted between stages 2 and 4. During this period there was no significant change in the concentration of $\sim$P (Fig. 2(b)), though the rise in the mean between stage 2 and stage 4 is only just short of
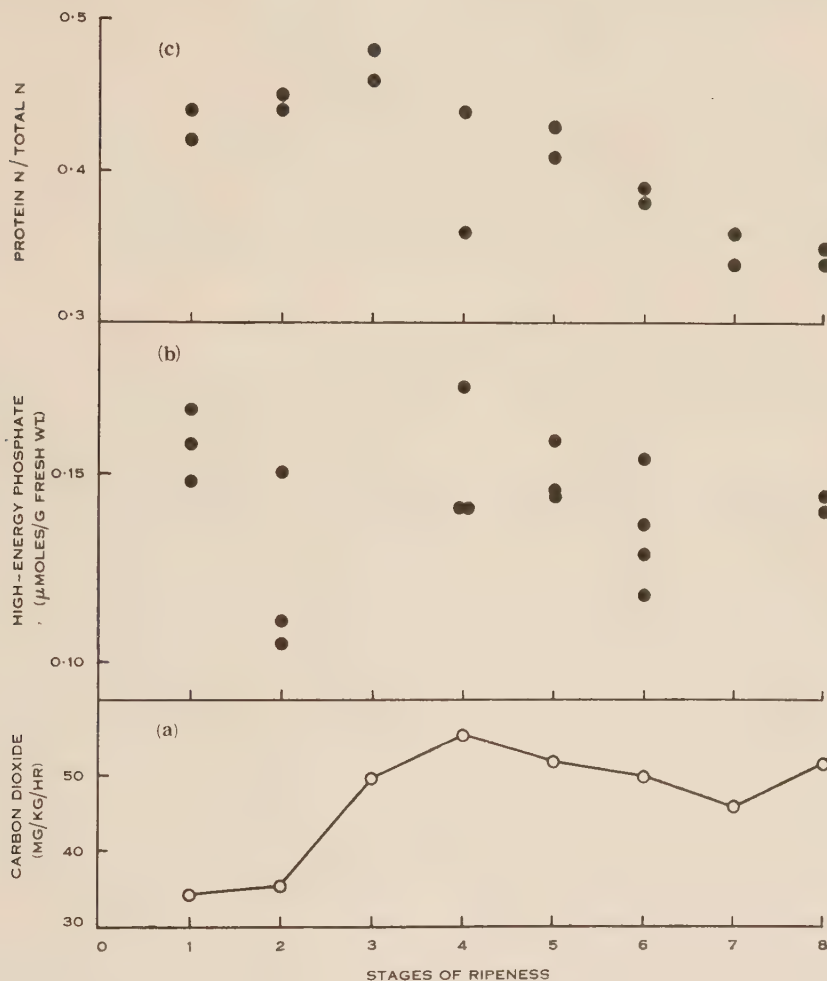


Fig. 2.—Changes in tomato fruit with stage of ripeness. (a) Rate of respiration per gram fresh weight; (b) concentration of high-energy phosphate per gram fresh weight; (c) ratio of protein nitrogen to total nitrogen.

being significant. After the peak in respiration, no significant change in concentration of $\sim$P was observed. The ratio protein nitrogen/total nitrogen (Fig. 2(c)) increased in the first three stages and then decreased steadily in the later stages.

These results present some contrast to the results for the avocado. There is no spectacular increase in the concentration of $\sim$P with the large increase in the

rate of respiration, and the ratio protein nitrogen/total nitrogen falls, although the rate of respiration remains high and the concentration of $\sim$P does not change.

## IV. Discussion

The results with the two fruits, avocado and tomato, suggest that the relationships of energy-rich phosphate, respiration, and protein levels are not uniform. In avocado, ripening after picking, $\sim$P increased with respiration, and the ratio protein nitrogen/total nitrogen increased throughout the experiment. In tomato, ripening on the plant, $\sim$P did not rise significantly at the climacteric. The ratio protein nitrogen/total nitrogen increased to a maximum at one stage of ripeness before the maximum rate of respiration and decreased progressively in the later stages of ripeness. Marks *et al.* (1957) reported a marked decrease in the rate of esterification of $^{32}$P after the climacteric. This is consistent with the steady state concentration of $\sim$P shown in the present study only if the ratio ADP/ATP increases as the fruit matures.

The measurements of $\sim$P reported here, and the work of Marks *et al.* suggest that the rise in respiration is probably not to be interpreted in the manner preferred by Millerd *et al.* (1953) because if uncoupling were taking place the concentration of $\sim$P would be expected to fall as the respiration increased. The increase in $\sim$P observed is consistent with the failure of dinitrophenol, in the work of Millerd *et al.*, to stimulate the respiration of tissue slices at the time of the climacteric, because the relative effect of dinitrophenol would be less when more ATP was being synthesized.

Pearson and Robertson (1954) suggested that the climacteric in apple fruit might be due to an increase in ADP. In the experiments reported here, ADP was not measured, but as $\sim$P increased at the climacteric in avocado, it is probable that the concentration of ADP increased also. Such an increase in ADP would account for the increased respiration. Howard, Yamaguchi, Pratt, and Rowan (unpublished data) have recently observed an increase of ADP during the climacteric in ripening tomato fruit. During the period of increasing respiration, the ratio protein nitrogen/total nitrogen also increased, so increase in ADP might be due to, in part, increased rate of protein synthesis. After the climacteric, the avocado differed from the tomato because the ratio protein nitrogen/total nitrogen remained high, though the respiration rate fell markedly and $\sim$P fell slightly.

While certain consistent changes have been noted, the initial cause of the increases accompanying the climacteric remained unexplained. It is likely that the rise in the respiration which has been called the climacteric and is associated with ripening has different causes in different fruits. The complexities may become obscured by discussions about "the cause" when causes are what should be sought. Some of the possibilities and the limitations of the present evidence have been reviewed by Laties (1957).

The increase in synthetic activity and the respiration rate observed in our experiments reflect a change in the pattern of metabolism which is under the control of some factor yet unknown. When the rate of synthesis of protein rises, the concentration of a number of enzymes might rise, including those concerned in

respiration, and further changes associated with ripening may be induced. Tager and Biale (1957), for instance, have shown an increase in activity of carboxylase and aldolase in banana fruit passing through the climacteric. It is perhaps relevant that Turner and Turner (1957) have shown that a rapid synthesis of starch in the developing pea seed is associated with an increase in concentration of starch phosphorylase.

## V. Acknowledgments

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# THE INHIBITION OF PHOTOSYNTHESIS BY OXYGEN

## II. THE EFFECT OF OXYGEN ON GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE FROM CHLOROPLASTS

By J. S. Turner,* J. F. Turner,† K. D. Shortman,‡ and Judith E. King†

### Summary

(i) An extract containing glyceraldehyde phosphate dehydrogenases was prepared from the chloroplasts of silver beet. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) functioned as cofactors for the enzyme activity.

(ii) The activity of the glyceraldehyde phosphate dehydrogenases was a function of the concentration of added cysteine. The enzymes were inhibited by low concentrations of iodoacetate and *p*-chloromercuribenzoate.

(iii) The reaction catalysed by both the DPN- and TPN-linked dehydrogenase (in the presence of an appropriate concentration of cysteine) was inhibited by oxygen. The rates in nitrogen and air were not significantly different in most experiments, but there was always a marked inhibition in pure oxygen.

(iv) The possible significance of these observations on the inhibition of photosynthesis by oxygen is discussed.

## I. Introduction

In Part I of this series (J. S. Turner, Todd, and Brittain 1956) the primary data on the inhibition of photosynthesis by oxygen were assembled. The strongly marked inhibition is produced rapidly and equally rapidly reversed. At high $CO_2$ concentration the inhibition caused by 20 per cent. oxygen is not significant; maximal inhibition is caused by pure oxygen. The inhibitory effect is found in all the green plants tested and applies both to $CO_2$ uptake and oxygen output. It has not been demonstrated for the Hill reaction (Brittain, unpublished data), but has been found to apply to $CO_2$-fixation by whole plastids (Arnon, Allen, and Whatley 1954).

The inhibition is not due to the effect of oxygen on the dark respiration, but the means by which oxygen inhibits real photosynthesis has not yet been established. In Part III of this series the conditions under which the effect is realized will be described and several different explanatory hypotheses discussed. In the present paper we summarize results which suggest that oxygen may act on photosynthesis in the same way as does iodoacetate—causing an inhibition of the whole process

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by reacting with the sulphydryl-containing enzyme glyceraldehyde phosphate dehydrogenase (GPD).

The same mechanism has been proposed as one explanation of the Pasteur effect in plant respiration. During studies on sucrose synthesis, pea seed extracts were obtained which possessed a complete glycolytic system (J. F. Turner 1954, 1957). The glycolytic activity was higher under anaerobic than under aerobic conditions, and it was suggested that oxygen inhibited the glycolysis by inactivating one of the glycolytic enzymes (J. F. Turner and Mapson 1958). Subsequent investigations (Hatch and J. F. Turner, unpublished data) have shown that the decreased rate of glycolysis *in vitro* was due to inhibition of GPD by oxygen through oxidation of sulphydryl groups of the enzyme. It is not yet known whether such a mechanism operates *in vivo* in cells possessing a Pasteur effect (see J. S. Turner 1958).

Glyceraldehyde phosphate dehydrogenases, reacting with the two cofactors diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) occur in green leaves (Arnon 1952; Gibbs 1952). It will now be shown that the reaction catalysed by these enzymes obtained from chloroplasts is inhibited by high oxygen concentrations, and it is suggested that this is one possible mechanism for the inhibition of photosynthesis by oxygen.

## II. Materials and Methods

### (a) Substrates

Fructose 1,6-diphosphate was prepared by the method of Neuberg and Lustig (1942). DPN and TPN were obtained from Pabst Laboratories, Wisconsin, U.S.A.

### (b) Preparation of Chloroplast Extract

Chloroplasts were isolated from the laminae of leaves of silver beet (*Beta vulgaris* L. var. *cicla* Moq.) by the method of Arnon, Allen, and Whatley (1956). The chloroplast extract was prepared by suspending washed chloroplasts in water and centrifuging as described by Whatley *et al.* (1956). These authors have reported that chloroplast extracts contain GPD.

### (c) Assay of GPD Activity

GPD (both DPN- and TPN-linked) was assayed substantially as described by Gibbs (1955). Where effects of gas phase were not being investigated the following components were placed in a spectrophotometer cuvette: 100 $\mu$moles 2-amino-2-hydroxymethylpropane-1,3-diol (Tris)-HCl buffer, pH 8·45, 51 $\mu$moles sodium arsenate, 12 $\mu$moles cysteine, 60 $\mu$moles sodium fluoride, 1·41 $\mu$moles fructose 1,6-diphosphate, 0·3 ml chloroplast extract. The reaction was initiated by adding 0·2 $\mu$mole DPN (or TPN). The total volume of the enzymic digest was 3 ml. The reaction was followed by measuring the change in absorbance at 340 m$\mu$ in a Beckman model DU spectrophotometer.

In studying the effects of the gas phase on the enzyme activity, enzymic digests of the composition described previously (except that a lower concentration of cysteine was used) were placed in Thunberg tubes, the DPN (or TPN) being placed

in the stopper. The tubes were evacuated on a water-pump and refilled with the appropriate gas. This process was repeated twice and the tubes were allowed to stand at room temperature with occasional gentle agitation. After 1 hr the reaction was started by tipping the pyridine nucleotide from the stopper. At the appropriate reaction time, the tubes were opened and the absorbance at 340 m$\mu$ of the contents measured immediately.

### (d) Assay of Aldolase Activity

Aldolase was assayed by the method of Stumpf (1948). The enzymic digests contained, in a total volume of 4 ml, 100 $\mu$moles Tris-HCl buffer, pH 8·45, 20 $\mu$moles fructose 1,6-diphosphate, 250 $\mu$moles potassium cyanide, 1 ml chloroplast extract. The triose phosphates formed were decomposed with 1N NaOH at room temperature and the inorganic phosphate released determined by the method of Allen (1940). The effects of gas phase on aldolase activity were studied using Thunberg tubes as described for the assay of GPD; the reaction was started by tipping chloroplast extract from the stopper.

## III. Results

Preliminary experiments showed that glucose 6-phosphate dehydrogenase did not interfere with the assay of GPD in the chloroplast extract. Neither DPN nor TPN was reduced in the presence of 10 mM iodoacetate which inhibits GPD but not glucose 6-phosphate dehydrogenase (Gibbs 1955). There was no significant reduction of the nucleotides in the absence of added fructose 1,6-diphosphate indicating that oxidation of cysteine was not interfering in the assay of GPD. No significant change in absorbance at 340 m$\mu$ was observed when the enzymic digests were incubated without added DPN or TPN.

### (a) Aldolase Activity

As the chloroplast extracts contained aldolase, fructose 1,6-diphosphate could be used as substrate for GPD. There was no difference in enzyme activity when digests of the composition described for the assay of aldolase were incubated under oxygen and nitrogen. Any effect of oxygen on the GPD assay was therefore not due to an effect on aldolase but on GPD.

### (b) GPD Activity

(i) *Effect of Cysteine.*—In the absence of added cysteine, there was no DPN-linked GPD activity in the chloroplast extracts in air. With some chloroplast preparations there was a small TPN-linked GPD activity without added cysteine, but the addition of an optimal amount of cysteine increased this rate by at least 10 times.

The effect of cysteine concentration on DPN-linked GPD activity is shown in Table 1. The critical concentration of cysteine lay between 0·4 and 0·04 mM. Cysteine concentration had a similar effect on the activity of TPN-linked GPD (see Table 1).

(ii) *Inhibition of GPD by Iodoacetate and* p-*Chloromercuribenzoate.*—GPD activity was very sensitive to iodoacetate. Both the DPN- and TPN-linked enzymes

were completely inhibited by 10 mM iodoacetate. The DPN-linked system was inhibited by 98 and 40 per cent. by 1 mM and 0·1 mM iodoacetate respectively,

TABLE 1

EFFECT OF CYSTEINE CONCENTRATION ON DPN- AND TPN-LINKED GLYCER-
ALDEHYDE PHOSPHATE DEHYDROGENASE (GPD)

Enzymic digests were of the composition described for assay of GPD (see text)

| Coupling | Cysteine Concn. (mM) | Incubation Time (min) | Increase in Absorbance at 340 m$\mu$ |
|---|---|---|---|
| DPN-linked | 4 | 54 | 0·219 |
| | 2 | 54·5 | 0·213 |
| | 0·4 | 55 | 0·222 |
| | 0·04 | 48 | 0·016 |
| | 0·004 | 49 | 0·011 |
| | 0 | 49·5 | 0·001 |
| TPN-linked | 4 | 32 | 0·146 |
| | 0·4 | 32·5 | 0·138 |
| | 0 | 33 | 0·007 |

and the TPN-linked system by 100 and 66 per cent. $p$-Chloromercuribenzoate (0·3 mM) completely inhibited both DPN- and TPN-linked GPD; with 0·1 mM

TABLE 2

EFFECT OF OXYGEN AND NITROGEN ON DPN- AND TPN-LINKED GLYCER-
ALDEHYDE PHOSPHATE DEHYDROGENASE (GPD)

Enzymic digests were of composition described for assay of GPD (see text)

| Coupling | Cysteine Concn. (mM) | Incubation Time (min) | Increase in Absorbance at 340 m$\mu$ | |
|---|---|---|---|---|
| | | | Nitrogen | Oxygen |
| DPN-linked | 0·4 | 15 | 0·330 | 0·013 |
| | 0·2 | 23 | 0·252 | 0·044 |
| | 0·1 | 30 | 0·023 | 0·051 |
| TPN-linked | 0·4 | 30 | 0·237 | 0·179 |
| | 0·2 | 30 | 0·226 | 0·083 |
| | 0·1 | 30 | 0·017 | 0·012 |

$p$-chloromercuribenzoate, the TPN-linked enzyme was unaffected and the DPN-system was inhibited by approx. 50 per cent. These experiments were carried out in the presence of 0·4 mM cysteine.

(iii) *Effect of Aerobic and Anaerobic Conditions on GPD Activity.*—In experiments designed to show a difference in GPD activity under aerobic and anaerobic conditions, a concentration of cysteine was chosen which would barely sustain maximum GPD activity in air. In preliminary experiments, it was found that no consistent difference could be demonstrated between the reaction rate in air and nitrogen. In subsequent experiments oxygen was used instead of air.

The effect of incubation in oxygen and nitrogen on DPN-linked GPD activity in the chloroplast extracts is shown in Table 2. Depending on the cysteine level GPD activity could be inhibited by over 90 per cent. in an atmosphere of oxygen compared with anaerobic conditions. The maximum effect was shown when the final concentration of cysteine was 0·4 mM. The rates of GPD activity in oxygen were barely significant. At the lowest concentration of cysteine used (0·1 mM) GPD activity under nitrogen was very small.

Table 2 also shows the effect of oxygen and nitrogen on TPN-linked GPD activity. The effects were, in general, similar to those described for the DPN-linked GPD. The maximum effect was obtained in the presence of 0·2 mM cysteine and here the inhibition by oxygen was in excess of 60 per cent.; in other experiments inhibitions of over 90 per cent. were obtained. The inhibition by oxygen with 0·4 mM cysteine was not as effective. Enzyme activity with low cysteine concentration was again barely significant in both nitrogen and oxygen.

## IV. DISCUSSION

It is known that the glyceraldehyde phosphate dehydrogenases from higher plants (like those of yeast and animal tissues) are affected by sulphydryl reagents (Gibbs 1952). In the present work this has been confirmed, GPD activity in chloroplast extracts being highly sensitive to iodoacetate and *p*-chloromercuribenzoate. The direct effects on these enzymes of aerobic and anaerobic conditions have not previously been studied, although Lipmann (1933) proposed that the Pasteur effect in yeast was due to the reversible oxidation of sulphydryl groups in dehydrogenases concerned in glycolysis. Engelhardt and Sakov (1943) have also suggested that the redox potential determines the activity of phosphohexokinase.

The present work has shown that the GPD activity of chloroplast extracts, (both DPN- and TPN-linked), may be inhibited by high oxygen concentration. This is probably due to the oxidation of sulphydryl groups of the enzyme protein, but the exact nature of the mechanism has not been established. The results obtained may, of course, be due to a direct effect of oxygen (or iodoacetate), not on the enzyme protein but on a soluble sulphydryl compound present *in vivo* and corresponding to the cysteine which forms a part of the enzyme digest in our experiments.

Iodoacetate and iodoacetamide inhibit the respiration of green cells (Arnon 1952; Hölzer 1954) and are presumed to act on GPD in so doing. It has, however, long been known that the oxygen output in photosynthesis is even more sensitive to these reagents than is respiration (Kohn 1935; Hölzer 1954). Stepka (in Calvin *et al.* 1951) showed that iodoacetamide also inhibited the uptake of $^{14}CO_2$ by 90 per

cent. Arnon, Allen, and Whatley (1956) have shown that $CO_2$-fixation in green plastids is inhibited by iodoacetamide, arsenite, and *p*-chloromercuribenzoate. The Hill reaction is not inhibited by these sulphydryl reagents. Arnon *et al.* therefore argue that the sulphydryl compound 6-thioctic acid does not take part in photolysis and that sulphydryl compounds are concerned not with the early but with later phases of photosynthesis.

All these observations lend support to the "assumption that the next step in photosynthesis after the formation of 3-phosphoglyceric acid is its reduction to triose by an iodoacetamide-sensitive hydrogenase. To a certain extent these observations also make it plausible that the H donor in this reduction is a pyridine
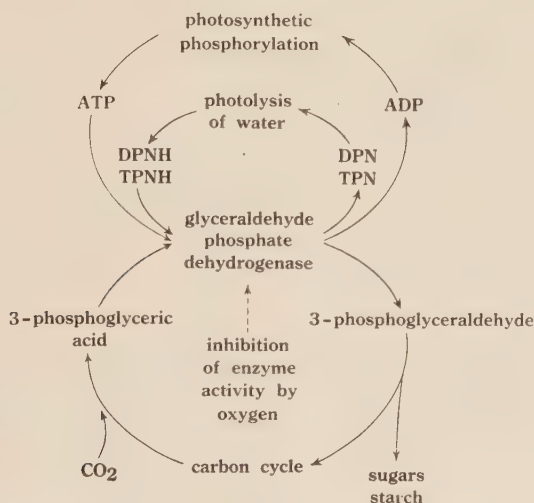


Fig. 1.—Suggested scheme for inhibition of photosynthesis by oxygen.

nucleotide and that ATP must be supplied to make the reduction possible" (Rabinowitch 1956). Such a scheme for photosynthesis (based largely on recent work in Arnon's school) is shown in Figure 1. The possible role of oxygen as an inhibitor, suggested by the results of the present investigation, is indicated. If GPD acted as a pacemaker in photosynthesis (e.g. at high light intensity and high $CO_2$ concentration) then inhibition of the enzyme by oxygen could inhibit photosynthesis (i) by depressing the rate of re-oxidation of reduced pyridine nucleotides derived from the photolysis of water, so that this process and oxygen evolution would slow down; (ii) by causing a block at the 3-phosphoglyceric acid level which could interfere with the operation of the carbon cycle.

Further work with plastids is in hand to test the hypothesis; meanwhile it may be pointed out that the effects of both oxygen and iodoacetate are greatest at saturating light, and that oxygen inhibits both photosynthesis (at high $CO_2$ concentration) and GPD only at very high oxygen concentrations.

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# THE MOVEMENT OF LEAF MAGNESIUM DURING THE GROWTH OF WHITE CLOVER PLANTS*

By T. F. Neales†

*Summary*

The time drift of the magnesium content of the leaflets of white clover was followed in plants transferred from a nutrient solution containing all the essential elements to one in which magnesium was omitted.

The magnesium content of leaflets formed before the transfer to the medium lacking magnesium did not diminish during 18 days subsequent growth in the absence of magnesium. There was no significant translocation of magnesium from the older leaflets (Mg content > 0·345 per cent.) to the younger leaflets (Mg content < 0·084 per cent.) formed on stolons after the transfer to the magnesium-deficient medium.

Evidence is cited demonstrating that the appearance of magnesium deficiency symptoms in the older, and not in the younger, leaves of a plant does not in itself constitute evidence of the mobility of magnesium within the plant.

## I. Introduction

The mobility or redistribution of the essential mineral elements after they have been incorporated into plant tissues has sometimes been inferred from the age of leaves in which deficiency symptoms of the element in question first appear. Thus, Wallace (1949, p. 11), from this type of evidence, states that magnesium seems to be very mobile within the plant. On the other hand, Ruck and Gregory (1955) have shown that there is little movement of magnesium from mature, rooted potato leaves to the shoot arising in the axils of these leaves. This result might be attributed either to the immobility of the magnesium incorporated into the mature potato leaves or to the lack of vascular connection between the potato leaf and the axillary bud it subtends. The experiments described in this paper were designed to ascertain if the magnesium in the leaves of white clover plants, grown in nutrient cultures amply supplied with magnesium, was translocated to young leaves formed after the plants were transferred to culture solutions not containing magnesium.

## II. Methods and Materials

Nodulated S100 white clover (*Trifolium repens* L.) plants were grown from seed in sand and, at the four-leaf stage, were transferred to nutrient cultures containing 100 p.p.m. Mg. The remaining essential nutrients were supplied according to a Long Ashton formulation (Hewitt 1952, p. 189). Three plants were allocated

to each 300-ml container, of which there were 30, and the plants were grown on in a heated greenhouse for 20 days. Each plant then had about 12 fully expanded trifoliate leaves. Eighteen containers were then matched visually for uniformity (the remaining 12 were discarded), and were randomly divided into the two lots of nine each, designated treatments A and B respectively. The nine A containers were refilled with the nutrient solution containing 100 p.p.m. Mg, whilst the B containers were refilled with a solution in which $MgSO_4.7H_2O$ had been replaced by $Na_2SO_4$. The concentration of the sulphate radical was the same in both nutrient solutions. The nutrient solutions were changed weekly.
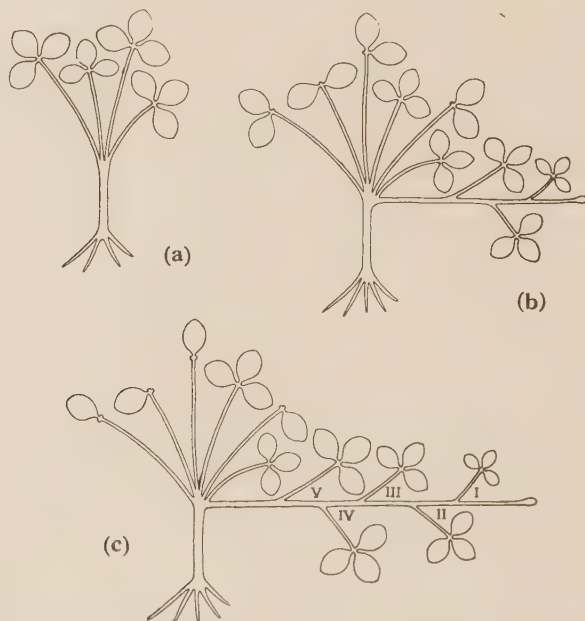


Fig. 1.—Diagrammatic representation of clover leaflet sampling technique (see text). (a) Before removal of first leaflet sample; (b) before removal of second leaflet, showing one stolon only; (c) before removal of third leaflet, also showing the numbering of the stolon leaves.

*Sampling methods.*—These "plus" and "minus" treatments were imposed on March 19, 1956, and also on this day a single leaflet of all the expanded leaves was cut off and dried, the leaflets from the plants of each container being kept separate. On this and all other occasions the samples were taken at 10 a.m. The dry weights and magnesium contents of the leaflets of each sample were measured. Second and third samplings of the leaflets of leaves fully expanded on March 19 were taken on March 30 and April 6, 1956. The leaflets were taken at random with respect to their position on the trifoliate leaf on each occasion (Fig. 1). These data enabled the comparison to be made of the time drifts of the magnesium content of the leaflets of plants of treatments A and B for the 18 days of the experiment. This method of leaflet sampling is similar to that of Ruck and Gregory (1955) and, as they point out,

its accuracy depends upon the high correlation, which they demonstrated, between the mineral content of paired leaves. The method further depends upon the assumption that the removal of one leaflet does not influence the magnesium metabolism of the remaining two.

During the experimental period, March 19 to April 6, the growth of the stolons of the clover plants was vigorous, and each stolon produced up to five expanded leaves. These five leaves were designated I to V, according to their position on the stolon, I being the distal, and, therefore, the youngest expanded leaf (Fig. 1).

TABLE 1

EFFECT OF MAGNESIUM SUPPLY ON THE DRY WEIGHT AND MAGNESIUM CONTENT OF CLOVER LEAFLETS

Each value is the mean of nine replicates

| Date of Sampling | Leaflet Dry Wt. (mg) | | Mg Content ($\mu$g per leaflet) | | Mg Concentration (% dry matter) | |
|---|---|---|---|---|---|---|
| | +Mg | −Mg | +Mg | −Mg | +Mg | −Mg |
| 19.iii.56 | 2·05 | 1·92 | 9·7 | 9·4 | 0·48 | 0·49 |
| 30.iii.56 | 2·55 | 2·56 | 14·7 | 9·2 | 0·58 | 0·36 |
| 6.iv.56 | 2·66 | 2·64 | 18·5 | 9·1 | 0·70 | 0·35 |
| Least significant difference (P = 0·05) | 0·33 | 0·22 | 2·5 | 0·5 | 0·05* | 0·04* |

*Calculated from logarithmic transformations.

On April 10, six stolons were selected from the clover plants growing in each container and the complete trifoliate leaves (without the petioles) of positions I–IV were harvested separately. These leaves were dried at 90°C, weighed, and their magnesium content measured by a modification of the method of Mason (1952). These data enabled comparisons to be made between the distribution of magnesium and dry weight of stolon leaves formed during 22 days growth in the presence or absence of magnesium in the culture solutions. The sampling technique used is illustrated in Figure 1.

### III. RESULTS AND CONCLUSIONS

#### (a) Appearance of Magnesium Deficiency Symptons

A slight chlorosis indicative of incipient magnesium deficiency was apparent 15 days after the transfer of plants to the solutions lacking magnesium, and 2 days later (April 5) the typical necrotic symptoms were visible. It was very noticeable, however, that these symptoms were apparent only in those leaves the greater part of whose development took place after the transfer to the solutions lacking magnesium. The majority of the affected leaves, therefore, were borne on stolons. The youngest

of these leaves on a stolon (I) was fully green, and the severity of the deficiency symptoms increased progressively from leaf II to leaf IV (Plate 1).

### (b) *The Effect of Sampling Time on the Leaflet Dry Weight and Magnesium Content of Leaves Fully Formed on March 19, 1956*

The three sampling times were separated by 11 and 7 days respectively. The mean increase in the dry weight (28·6 per cent.) of the leaflets of both treatments from March 19 to March 30 indicates (Table 1) that the leaflets were immature on March 19. There was no significant increase in dry weight over the second sampling period. This increase in leaflet dry weight over the first sampling period entirely

TABLE 2

DRY WEIGHTS AND MAGNESIUM CONTENT OF STOLON LEAVES I–IV

Each value is the mean of nine replicates

| | | Leaf Number | | | | Least Significant Difference ($P = 0.05$) |
| --- | --- | --- | --- | --- | --- | --- |
| | Treatment | I (youngest) | II | III | IV | |
| Mean leaflet dry weight (mg) | +Mg | 4·55 | 6·51 | 6·36 | 4·87 | 0·79 |
| | −Mg | 2·87 | 5·13 | 6·45 | 6·85 | 0·53 |
| | | $P<0.001$ | $P<0.001$ | n.s. | $P<0.001$ | |
| Mean leaflet magnesium content ($\mu$g) | +Mg | 12·0 | 18·0 | 15·0 | 12·3 | 2·21 |
| | −Mg | 2·4 | 4·0 | 3·1 | 3·4 | 0·46 |
| Magnesium concentration in stolon leaves (% dry wt.) | +Mg | 0·264 | 0·277 | 0·236 | 0·253 | |
| | −Mg | 0·084 | 0·078* | 0·048* | 0·049* | |

*Showing magnesium deficiency symptoms.

accounts for the decrease in magnesium concentration in the leaflets of the treatment B plants. It is evident that there is no significant loss of magnesium from these leaflets.

Williams (1955) has pointed out that direct quantitative evidence for the redistribution of magnesium within the plant is scanty. Wallace (1949) claims that there is considerable redistribution of magnesium from old to young leaves in plants growing under conditions of magnesium shortage, and Phillis and Mason (1942) arrived at a similar conclusion. Ruck and Gregory (1955), however, reported that magnesium was not readily transferred from the leaflets of rooted potato leaves to the tissues of a shoot arising from the bud subtended by each leaf. The evidence

presented above suggests no redistribution of magnesium from mature leaves of intact white clover plants.

### (c) *Magnesium Content of the Leaves of Clover Plants Formed after the Transfer to Magnesium-deficient Solutions*

On April 10, 22 days after the clover plants of treatment B had been transferred to culture solutions lacking magnesium, the stolon leaves formed during this period were sampled as previously described. The mean dry weight and magnesium content of leaves of positions I–IV are given in Table 2. These results have been presented on a leaflet basis to facilitate comparisons with Table 1.

These results indicate that the leaflets of plants growing in solutions supplied with magnesium contained about four times as much magnesium as those of the magnesium-deficient plants, irrespective of leaf position. The origin of the magnesium which does enter the stolon leaves formed after the transfer to magnesium-deficient solutions is uncertain. However, it has been shown above that the older leaves, containing quantities of magnesium considerably in excess of that required to prevent chlorosis, do not lose magnesium to the younger leaves. It is probable that there remains, possibly within the stem and root xylem, a proportion of the total magnesium content within the plant that is able to be translocated and support new growth after the removal of magnesium from the root environment. Alternatively, the magnesium may move more readily from stems and roots than from mature leaves.

Examination of Table 2 shows that magnesium deficiency symptoms appeared in the three older stolon leaves which had a higher magnesium content, but a lower magnesium concentration, than the youngest leaf (I) which was fully green (Plate 1). It is apparent therefore that the appearance of magnesium deficiency symptoms depends upon the leaflet magnesium concentration, whilst evidence for the translocation of this element must come from changes in the total magnesium content of leaf. The results in Table 2 also show that the dry weight of the two youngest leaves of the magnesium-deficient plants was less than that of the corresponding leaves of the "plus" magnesium plants. This relationship was reversed in leaf IV.

### IV. DISCUSSION

Although it is apparent (Plate 1) that leaf II is severely deficient in magnesium whilst leaf I is healthy, the magnesium concentration in these two leaves is very similar (0·078 and 0·084 per cent.). Leaves III and IV exhibit symptoms of extreme magnesium deficiency and it is probable that, for the conditions of this experiment, 0·08 per cent. represents the threshold magnesium concentration below which magnesium deficiency symptoms appear. The suggestion is made therefore that in the fully green, but immature, leaf I there is a "physiologically active" component of the leaf magnesium of sufficient concentration to prevent chlorophyll degradation. Garner *et al.* (1930) have concluded that, in order to prevent the breakdown of chlorophyll, the total magnesium content of the tobacco leaf must be several times the quantity present in the chlorophyll. It is further envisaged that, as the dry

weight and incorporation of cell wall material increase, an increasing proportion of the leaf magnesium is "inactivated" by inclusion in the structural material of the leaf, and chlorophyll degradation proceeds, as in leaf II. In support of the above hypothesis, it is well known that magnesium accounts for a considerable proportion of the metallic content of the pectates of the primary cell wall (see, for instance, Ehrlich and Sommerfeld 1926); and also it has been shown (Neales 1956) that 62 per cent. of the magnesium of mature white clover leaves is found in the colourless fibrous residue remaining after maceration and repeated extraction with 80 per cent. aqueous acetone.

Thus, when the leaf is growing under magnesium starvation conditions, there is little or no addition to the "active" component of leaf magnesium by translocation, chlorophyll degradation proceeds, and the chlorosis typical of magnesium deficiency appears. No such symptoms appear in the control plants adequately supplied with magnesium because there is a continual supply of magnesium by uptake from the nutrient solution.

## V. Acknowledgments

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LEAF MAGNESIUM MOVEMENT
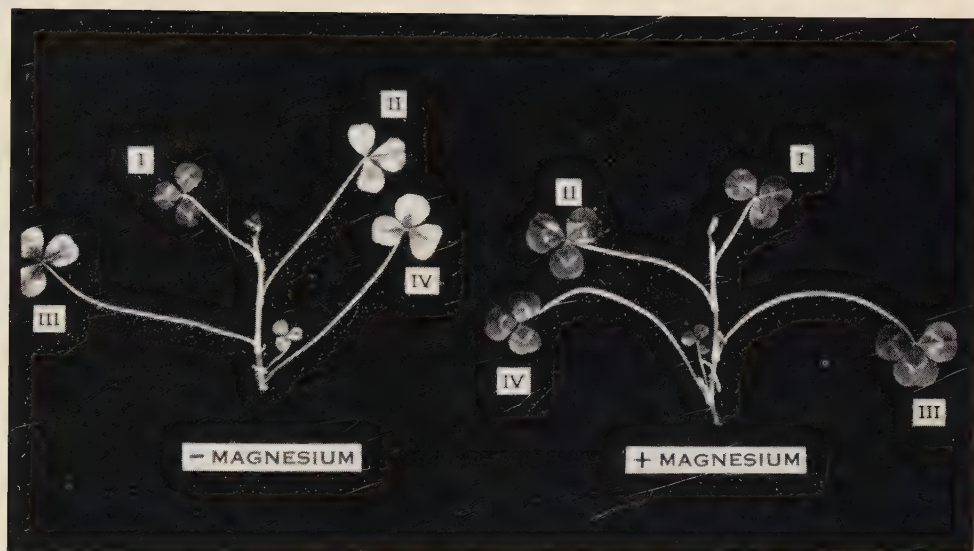


Stolons of white clover on April 10, 1956, after 22 days growth in the absence or presence of magnesium in the nutrient solution.

# THE MEASUREMENT OF DIFFUSION PRESSURE DEFICIT IN PLANTS BY A METHOD OF VAPOUR EQUILIBRATION

## By R. O. Slatyer*

### Summary

A method for measuring diffusion pressure deficit (DPD) in plants is described and compared with the standard floating techniques. Basically the method is a modification of that described by Arcichovskij and Arcichovskaja (1931) and differs from the standard floating methods in that it involves the equilibration of tissue in vapour of known water potential rather than in solutions of known osmotic potential.

The technique involves the use of micro-desiccators in which disks of leaf tissue are arranged on small grids 5 mm above the surface of salt solutions of known water potential. Equilibration takes place in a constant-temperature water-bath, with the temperature controlled to $\pm 0.001°C$.

In a comparison with the standard floating methods, the vapour technique is shown to have advantages in precision and reproducibility at all degrees of water stress above a few atmospheres DPD. At these low stress levels both methods appear satisfactory but, as turgor pressure falls with increasing stress, errors begin to be introduced to the floating methods by cell plasmolysis and at the stage when most of the cells in the tissue are plasmolysed the method becomes almost completely unresponsive.

## I. Introduction

The measurement of diffusion pressure deficit (DPD) (Meyer 1945) in plant tissues has been the subject of a great deal of study extending from the early investigations of de Vries (1884). The principal technique which has been evolved is that which involves balancing the DPD, which is equivalent to the water potential† of the plant tissues, against the water potential (or osmotic potential) of aqueous solutions of sucrose, mannitol, or salts, using a series of solutions of differing concentration. The solution in which the tissue neither gains nor loses water from or to the solution, respectively, is considered to have an osmotic potential equivalent to the DPD of the tissue. Examples of this general technique are the cell method (Ursprung and Blum 1916) in which change in cell volume is measured, the strip or simplified method (Ursprung 1923) in which change in length or volume (Lyon 1936) is measured, and the weight method (Meyer and Wallace 1941) in which the change in weight is measured. An alternative measurement (Ashby and Wolf 1947) involves a determination of change in the concentration of the solution, using a refractometer.

Although this technique has great simplicity, the need to float tissue in the solution can introduce appreciable errors due to infiltration of the solution into the intercellular spaces, and in the case of plasmolysis, intracellular spaces, of the tissues.

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†Terminology of Schofield, cited by Owen (1952).

As a result, interest has been focused on vapour pressure methods for measuring DPD. Two main techniques suggest themselves. The first is to utilize the same general procedure as has just been described, but to balance the DPD of the tissues against the water potential of vapour over a solution, instead of directly against the osmotic potential of a solution. The second is to measure a characteristic of the vapour pressure when the tissue under study is enclosed in a thermally constant chamber. An example of the first technique is the method of Arcichovskij and Arcichovskaja (1931) which measures the change in weight of samples of leaf tissue which are placed in vapour over solutions of known water potential. Examples of the latter technique are the methods of Spanner (1951) who used an application of the Peltier effect to measure wet-bulb depression and hence to compute water potential, and of Stone, Went, and Young (1950) who employed a humidity-sensing device to effectively measure water potential under conditions of extreme water stress.

The latter methods have some advantages over the former in speed and precision and it can be anticipated that, as more precise methods of hygrometry are developed, still more applications will follow. In most biological laboratories at present, however, the necessary equipment is not readily available and for this reason, and because of the simplicity of the former method, it was decided to investigate it with a view to improving its accuracy and flexibility.

## II. Apparatus and General Technique

The principal requirement of the apparatus was that it should provide and maintain, at the surface of the tissue under examination, a constant and known water potential in the vapour phase. This necessitated very sensitive temperature control and the use of small test chambers, so that differences in water potential at the surface of the control solution and at the tissue surface would be as small as possible.

### (a) Control of Water Potential

(i) *Constant-humidity Chambers.*—These chambers were constructed from standard laboratory pomade jars about 6 cm high and 6 cm diameter, fitted with completely watertight rubber-lined plastic lids. The jars were filled to within 1·2 cm of the top with coarse quartz sand or glass beads to minimize splashing of solution during manipulation of the chambers. The chambers were then filled with control solution so that the solution covered the sand and reached to within 1·0 cm of the top. A small grid of nylon mesh which was to carry the plant material was then arranged 0·5 cm above the liquid surface, supported on a short (2 cm) length of 2-cm diameter glass tubing which was pushed into the quartz sand to the desired depth. The tubing also acted as a baffle to prevent splashing. A diagrammatic sketch of the completed chamber is shown in Figure 1.

(ii) *Control Solutions.*—Precise data on the aqueous vapour pressure of pure water, and of solutions of sodium chloride, are available together with data on the relative molal vapour pressure lowering $[(P_0-P)/MP_0]$ for various concentrations of sodium chloride (Robinson and Stokes 1955). The equivalent values for the water

potential in centimetres of water were obtained from the formula provided by Owen (1952):

$$d = \frac{RT}{gM}\left(-\log_e P/P_0\right),$$

where

$d$ = water potential in centimetres of water,
$R = 8 \cdot 314 \times 10^7$ ergs per °C per mole,
$T$ = absolute temperature, and
$g = 980 \cdot 6$ dynes per gram.

As DPD is measured by being balanced against osmotic solutions or vapour of known water potential, it should be expressed in the same terms, i.e. in centimetres or metres of water potential. However, Meyer (1945) regards DPD as a unit of pressure, conceptually and dimensionally different from a unit of potential energy, and expressible in atmospheres. Although this contention can be disputed, the use of DPD as a term in plant physiology has come into widespread use. For this reason it has been considered necessary, though undesirable, in this paper to refer to DPD
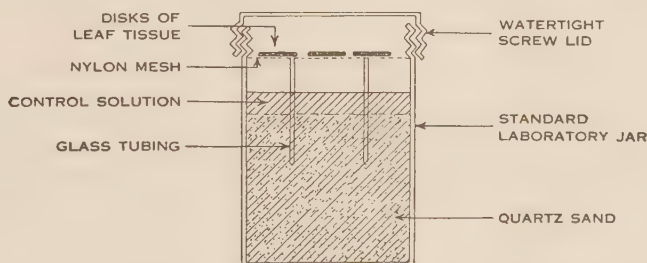


Fig. 1.—Diagrammatic sketch of constant-humidity chamber in which plant material was placed during determinations.

when discussing plant measurements, but to refer to the vapour in the controlled humidity chambers in terms of water potential. Though dimensionally incompatible, pressure and energy units can be regarded as numerically equivalent, and to this extent can be related by the expression:

water potential (in cm) = —1033 DPD (in atm).

The solutions were made up to the desired concentration and added to the constant-humidity jars as described above. No special arrangements were made for stirring of the solutions, but slight vibration in the water-bath assisted in the elimination of the possible concentration gradients in the solutions in each jar which could have developed through evaporation or condensation of water at the liquid surface. Best results were obtained when the solutions were replaced at the end of each determination. The temperature for all determinations was 25°C.

### (b) Control of Temperature

For the maintenance of constant water potential in the humidity chambers, it was important that they should be placed in a temperature-controlled water-bath, where sudden changes in temperature could be eliminated and possible condensation of water at the tissue surface avoided. A water-bath arrangement as

finally utilized has enabled the control of temperature to within $\pm 0.001°C$. It consisted of an outer tank of about 200 l. capacity in which was placed a smaller tank of about 75 l. capacity. The outer tank was controlled to better than $\pm 0.05°C$ and this enabled the inside one to be controlled to $\pm 0.001°C$ without undue difficulty. The whole apparatus was located in a constant-temperature room which was held at $25 \pm 0.5°C$. Various thermoregulators were used for the inside tank, the most effective being a Fisher-Serfass "Micro-Set" differential thermoregulator operating through a Fisher-Serfass electronic relay. A "Sunvic" toluene–mercury thermoregulator operating through a proportioning head and "Sunvic" EA3T electronic relay was utilized for the outside tank. Each thermoregulator actuated a 200-W standard frosted light bulb to provide intermittent heating. Cooling, when required, was provided by a copper coil through which water flowed at about 20°C. The water pressure was controlled by a constant-head source, and rate of flow was adjusted arbitrarily by a valve on the inlet tube. Rapid circulation of water in each tank was provided by propeller or pump-type stirrers (the latter being more satisfactory) which enabled uniform temperature conditions to be maintained throughout the tank.

### (c) General Method

The main objective of the present study was to improve the general technique of Arcichovskij and Arcichovskaja (1931) which was described above. Accordingly, a series of experiments was conducted to study the time needed for equilibration of the tissue used, and the influence of various factors which could affect the validity of the determinations. In general, several main procedures were always adhered to. Firstly, all plant material was allowed to reach temperature equilibrium in the constant-temperature laboratory before it was sampled. Secondly, the sample taken consisted of disks of tissue of varying size (see below) which were punched from the leaves using a sharp cork borer. The leaves were always selected for uniformity and representative age. After sampling, the disks were immediately transferred to tared weighing bottles and weighed on an automatic balance to 0·1 mg. They were then transferred to the constant-humidity chambers which remained in the water-bath except for the introduction or removal of samples. Each of these latter processes took about 40 sec per sample. At the end of each determination the disks were again transferred to the weighing bottles and reweighed. If any floating treatment was imposed, the disks, after removal from the liquid, were placed on filter paper to allow rapid drainage of adherent water and then dried for 30 sec between eight sheets of Whatman No. 4 filter paper under a 500-g weight. This procedure was adopted to ensure uniformity in the drying operation, a potential cause of serious weighing errors (Ashby and Wolf 1947).

The number of disks per sample varied with disk size and the degree of accuracy needed. The size of disk most frequently used was 0·7 cm diameter. Normally a sufficient number of disks was punched to bring the total weight of the sample to at least 0·10 g. Notwithstanding this provision, when larger disks were used, a minimum of six disks generally appeared necessary to obtain valid results, and a sample size of 10 or 12 disks was utilized where possible.

Several species of plants were utilized for the determinations, particularly privet (*Ligustrum japonicum* Ait. and *L. lucidum* Ait.), tomato (*Lycopersicon esculentum* Mill.), saltbush (*Atriplex nummularia* Lindl.), and sorghum (*Sorghum vulgare* Pers.). In general, the results obtained with these different tissues were very similar, and the data presented below are from privet and tomato, with which the major part of the investigations were conducted. Because the investigations took place in three laboratories over a period of more than two years, a wide variety of plants within these species was sampled. For this reason the data from different sections of the results presented are not always strictly comparable.

## III. Results

### (a) *Time Needed for Equilibration*

Before directly examining the method of Arcichovskij and Arcichovskaja, it was considered important to examine aspects of water uptake and loss by leaf disks in vapour of different water potentials, so that the effect of such factors as disk size, disk placement, and water potential on the time needed for equilibration could be evaluated.
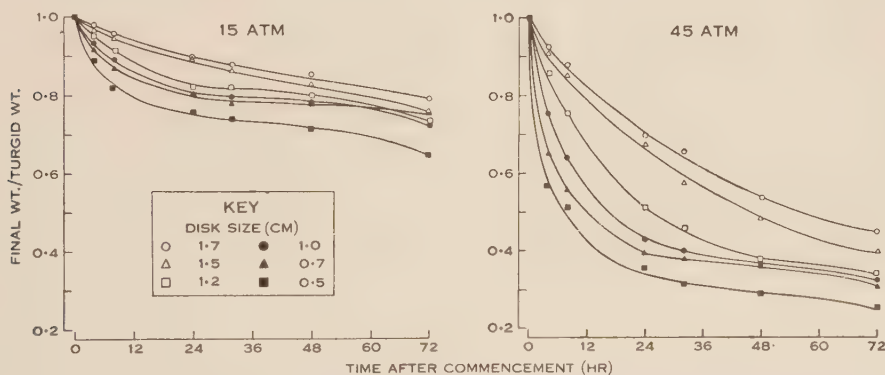


Fig. 2.—Effect of size of disk on rate of loss of water from tomato leaf tissue, in vapour of water potential equivalent to 15 atm and 45 atm DPD.

(i) *Effect of Disk Size.*—To investigate aspects of this effect, disks of various sizes, and hence of varying cut surface/total surface ratios, were used to determine the rate of loss of water in vapour of different water potential. Only the rate of loss was examined because of the difficulty in establishing uniform stress for the commencement of uptake. The disks were punched from unwilted plants, floated in water for 8 hr, dried, weighed, and placed in the appropriate constant-humidity chambers. In Figure 2, data for tomato tissue in water potentials equivalent to DPD's of 15 and 45 atm are presented which illustrate the pattern of response observed in all species at different water potentials.

The data show a rapid initial decline in weight followed by a transition to a final stage characterized by a slight but continuous weight loss. This final stage persisted to the end of the experimental period and probably reflected losses due to

respiration. The data also show that as disk size increased the rate of change in ratio of the final weight/original turgid weight increased, so that the final stage was reached fastest in the smallest, and slowest in the largest disks. This suggests that most of the water exchange takes place through the cut surfaces of the disks, the greater the cut surface/total surface ratio, the faster the rate of exchange.

Since the speed with which equilibration occurs is an important factor to be considered in assessing the effectiveness of vapour methods for measuring DPD, one of the objectives of this test was to indicate a disk size which would enable equilibration, or the attainment of the final stage, in the shortest period of time, but would not result in a degree of tissue damage severe enough to affect the final value. The 0·5-cm disks did not appear to satisfy the latter provision as they reached final values significantly lower than those of the other groups. Disks of greater than 1·2 cm diameter clearly failed to satisfy the former provision. Disks of the intermediate group were therefore indicated and after extensive preliminary tests with the species listed above, disks of 0·7 cm diameter were adopted, except where otherwise stated, for the remainder of the determinations reported in this paper.

(ii) *Effect of Water Potential.*—Two series of experiments were conducted in order to determine the rate of water uptake by, and loss from, leaf disks in vapour of different water potential.

In order to secure data on rates of both uptake and loss, branches were removed from the plants to be sampled and allowed to wilt very severely in the greenhouse. They were then brought into the constant-temperature room, allowed to equilibrate with room temperature, and two series of disks were punched from representative leaves. After weighing, one series of disks was immediately placed in the constant-humidity chambers; the other series was floated on water for 8 hr, dried, weighed, and then placed in the chambers. This enabled sorption* curves to be obtained from the former series and desorption curves to be obtained from the latter. The results for tomato and privet, for a range of water potentials, are shown in Figure 3. The sorption curves are not strictly comparable because it was not possible to create the same degree of stress in each species before sampling. The maximum DPD which could be developed in tomato without damage was about 40-50 atm; in privet, on the other hand, it was possible to develop well over 90 atm. As a result no data are given for tomato above 45 atm and in each species the rate of uptake is partly a reflection of the different initial degree of stress. The desorption curves are comparable because of the preliminary period of floating which enabled the disks to reach full turgor before being placed in the chambers.

From Figure 3 it is evident that the general rates of uptake were similar in both species, although slightly slower in privet. This also applied to rates of water loss, but this process was much more rapid. In general, with all the species tested, there was a tendency for water exchange to be slower in species with well-developed cuticles. As in the previous series of tests, final steady equilibrium values were not obtained, all the curves showing a slow decline after the final stage had been reached.

*Because both adsorption and absorption processes operate in the uptake of water by plant material, the term sorption is used in this paper to indicate uptake of water and desorption to indicate loss of water.

The rate at which the final stage was reached was markedly affected by water potential, particularly at values equivalent to DPD's of less than 15 atm. With the uptake curves, in the 0 atm treatment, the final stage did not appear to be reached in either species even after 72 hr. As the water potential increased negatively the time required decreased, but even in the most extreme cases more than 48 hr elapsed. In the loss process, which was much more rapid, the final stage was reached after about 24–32 hr at water potentials corresponding to DPD's in excess of 15 atm and after a little over 32 hr at lower values. Apart from differences in rate of equilibration, it is interesting to note that the final value obtained on the sorption curves always appeared to be less than the value on the desorption curve. This was possibly due to hysteresis and is discussed in more detail below.
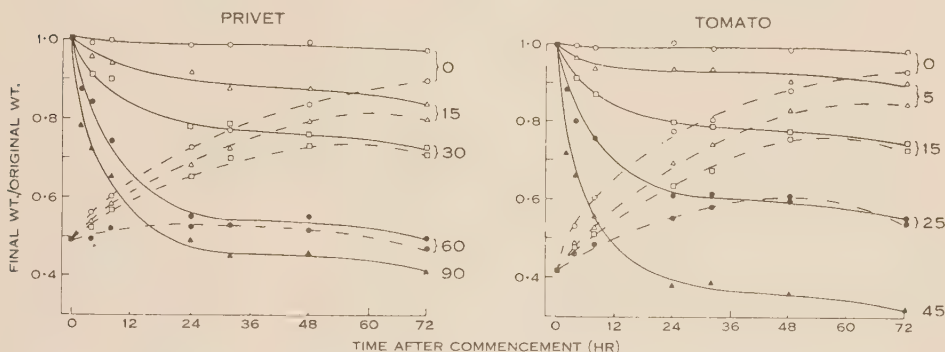


Fig. 3.—Change of weight of disks of privet and tomato leaf tissue in vapour of different water potential, in relation to time allowed for exchange of water by the disks; continuous lines represent loss, broken lines represent uptake. Water potential (in atm equivalent DPD) is indicated at end of each set of curves.

(iii) *Effect of Disk Placement.*—Because differences have been observed, in floating experiments, between disks placed with their adaxial surface uppermost, as distinct from those with abaxial surface uppermost, the possible influence of disk placement on rate of change of tissue weight, and on the final value obtained, was investigated. As before, only the loss curve was studied because of difficulties in standardizing the degree of stress before uptake. Disks were punched from unwilted plants and after floating and reweighing were arranged in the controlled humidity chambers so that one series had the adaxial sides of the disks uppermost and the other the abaxial sides uppermost. No significant differences were observed (at $P = 0.10$) regardless of the water potential involved or species used.

### (b) *Utilization of Method for DPD Measurements*

(i) *Time Needed for Determinations.*—Because final equilibrium values are extremely difficult to obtain, some arbitrary time limit must be set for the duration of DPD determinations. Such a period should be as short as possible, so as to minimize effects of respiration and because of the convenience, experimentally, of a rapid determination, but should be of adequate duration to minimize the effects of

differential rates of sorption and desorption, so as to provide a sensitive estimate of DPD.

In order to determine an appropriate period for the determination, samples were taken from plants which had previously been subjected to moderate–severe wilt. This procedure ensured that an appreciable water deficit developed in the tissue so that a significant amount of both uptake and loss could be expected during the determinations. After introduction to the constant-humidity chambers, different sets of samples were removed after 4, 8, 24, and 48 hr. The results obtained with privet and tomato leaf tissue, which were similar to those from the other species, are given in Figure 4.
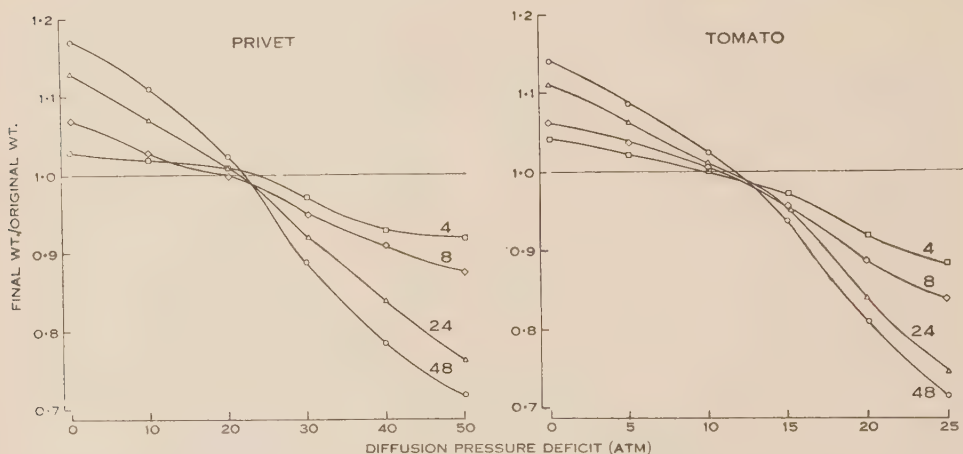


Fig. 4.—Influence of time allowed for determination of DPD of privet and tomato leaf tissue on the final value obtained. Time allowed (hr) shown on curves for each determination.

The data indicate that although a significant estimate of DPD could be made after 4 hr, the value obtained tended to·drift slightly with time. This appeared to be due primarily to slower initial rates of sorption, since with increasing time proportionally more uptake occurred and the point of intersection shifted along the line of final weight/original weight = 1·0. The difference between the various estimates can be seen to be of the order of 2–3 atm.

Although this suggests that as long a period as possible should be allowed for the determination, the data show that the uptake in the 24–48-hr period represents only a minor proportion of the total uptake, so that the advantages of extending the determinations beyond 24 hr are limited. Also, any undesirable effects due to respiration can be expected to become more pronounced with increasing time. For these reasons a 24-hr period was adopted as a standard time for the determination and has been found satisfactory in extensive laboratory use of the method.

(ii) *Factors Influencing DPD Value Obtained.*—From the data just presented it appears that the main factors which affected the value obtained for DPD in any one determination were those affecting the shape of the uptake and loss curves and so influencing the point of intersection with the line of final weight/original

weight $= 1.0$. As suggested above, the relatively slow rate of uptake was probably the primary factor in this regard.

In order to secure data on the extent to which these factors operated, it was decided to compare the results obtained with the standard method with those obtained when the tissue was first floated to turgor and then dehydrated to various degrees. This latter procedure should, in effect, examine only the desorption curve and so the amount by which the standard gain and loss curve diverged from it should be indicated.
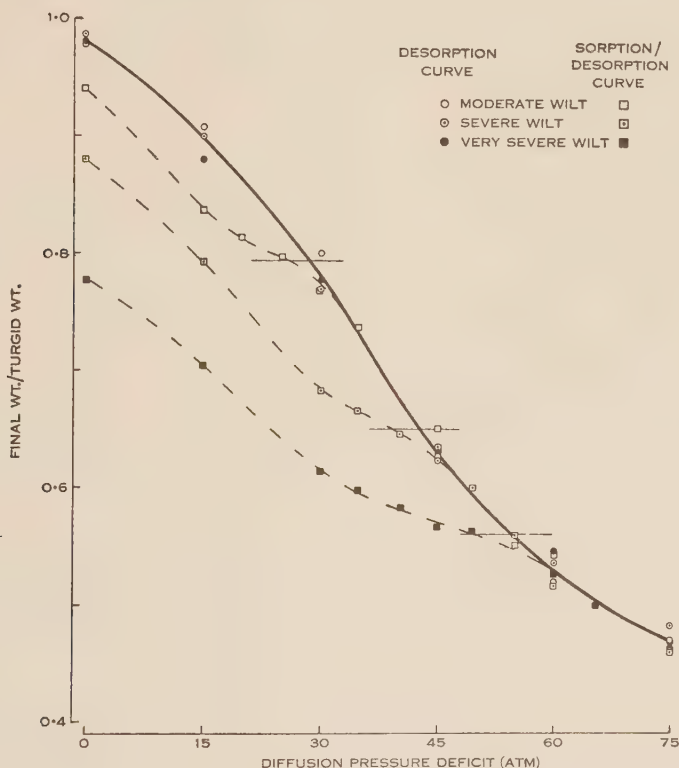


Fig. 5.—Comparison of value obtained for DPD of privet leaf tissue when determination is conducted by standard sorption/desorption technique, with value obtained from desorption curve after tissue has first been floated on water and then dehydrated to known degrees. Tissue first subjected to moderate, severe, and very severe wilting to develop a wide range of DPD's.

For these experiments, small branchlets of similar size and age were detached from sample plants and allowed to wilt to different stages so that different initial DPD's were developed. Two sets of disks were punched from the leaves and weighed in tared weighing bottles; one set was then placed immediately in containers comprising a range of known water potentials, the other was first floated in water for 8 hr and then dried, reweighed, and placed into a similar range of containers. The samples remained in the containers for 32 hr and were then removed and reweighed.

The results were plotted as final weight/turgid weight and are given for privet in Figure 5. Turgid weights for the samples not pre-floated were estimated from the original fresh weights, using the fresh weight/turgid weight ratios of the pre-floated samples. Short horizontal lines have been drawn across each curve at the level of the original fresh weight, so that the DPD can be read off at the point of inflexion.

The data indicate quite marked differences in the value obtained for the DPD, depending on the method used; the values obtained by using the desorption curve alone being up to 5 atm greater than those from the standard curve.

Although part of this difference might be attributed to incomplete desorption, it is apparent from the marked divergence of the two curves, which took place as soon as the sorption phase was introduced, that most of the difference seems to be caused by slow rates of sorption.

It is also possible that hysteresis effects could have contributed to the result. Although no direct evidence is available for such an explanation, the data of Figure 5 support such a contention. For instance, in the humidity chambers representing DPD's close to those existing in the tissue when sampled, the slower rates of uptake should not have been of significance in determining the final values obtained, since equilibrium should have been reached within the period allowed. Yet in such instances the final weight/turgid weight values on the sorption curves were significantly below those on the desorption curve. Such a phenomenon could be due to hysteresis, and on the basis of other studies with plant material (Rao, Rao, and Rao 1949) and with wood (Kelsey 1957) the effects of hysteresis could be expected to be of similar magnitude to the differences observed here. These points will be considered in the subsequent discussion.

From Figure 5 it is also interesting to observe the uniformity in all curves in the desorption phase. This suggests that there was little permanent effect on osmotic pressure of the tissue sap (and hence on the DPD/water content relationship) caused by the initial wilting or the pre-floating, and would tend to rule out the effects of such changes in the interpretation of the data in the diagram.

### (c) *Comparison of Vapour Equilibration Method with Floating Methods*

Crafts, Currier, and Stocking (1949) comment that the "simplified" or "strip" and "weighing" methods of measuring DPD (Ursprung and Blum 1930) are those most widely used at the present time. Yet it is undeniable that they are most unsatisfactory in all tissues when water stress becomes severe and even at quite low DPD's with fairly rigid tissue. In order to compare these established methods with the vapour method, comparative experiments were conducted using the normal techniques for the strip and weighing methods, and the standard technique as described in this paper for the vapour method (i.e. using 0·7-cm disks, with 24 hr equilibration, and assessing DPD as the estimated point at which there was no gain or loss of weight by the tissue). For the weighing method, 0·7-cm disks were used and change in weight was assessed after 2 hr floating in graded solutions of NaCl. For the strip method, different techniques had to be employed for the tomato and privet tissue. For tomato, 0·5 by 3·0 cm strips were used, change of length being measured after 2 hr floating, with a microscope and stage micrometer. For privet, change in

length was negligible and change in thickness was measured instead (Ursprung and Blum 1927) using a paper thickness measuring device, reading to 0·01 mm. The results are shown in Figure 6 for determinations made on tissue previously wilted to a slight, moderate, and severe extent.
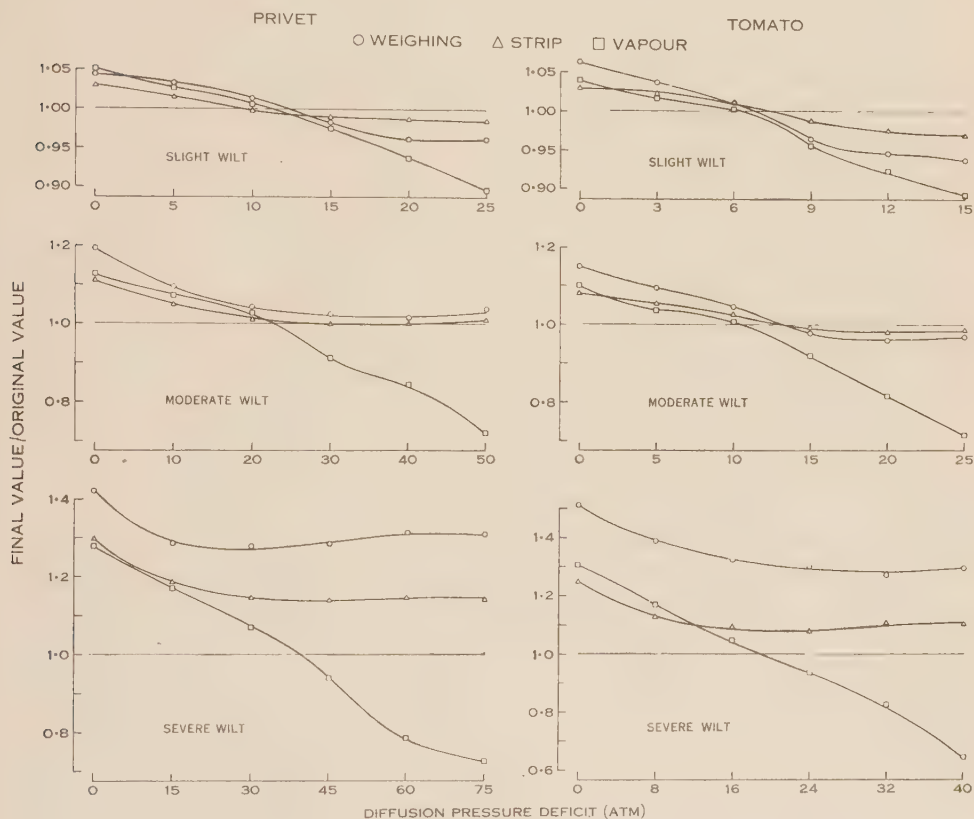


Fig. 6.—Comparison of standard vapour equilibration technique for determination of DPD with strip and weighing methods. The privet and tomato leaf tissue used was first subjected to slight, moderate, and severe wilting to develop a range of DPD's. The data are expressed as relative change in weight, length, or volume.

From the diagram it can be seen that in the slight wilt treatments, all methods gave estimates agreeing to within 2 atm, for both species. As the severity of wilting increased, however, the efficacy of the floating methods decreased, so that at moderate wilt no estimate was obtained by these methods for privet, and the estimate for tomato was much higher than that obtained by the vapour method. With the severe-wilt treatment no estimate was obtained, for either species, with the floating methods.

A probable explanation for this lack of effectiveness of the floating methods as stress increases is that infiltration of the external solution into the cells of the tissue takes place whenever the original DPD of the tissue is higher than that which

would exist at a state of incipient plasmolysis. Under these conditions it could be expected that, if the concentration of the external solution were greater than that of the plasmolytic value of the cell sap, infiltration by the solution would take place through the cell walls and each cell would swell to its shape at incipient plasmolysis. Some penetration could also possibly occur into the vacuole, and the final equilibrium situation would probably be one in which the protoplast became dehydrated to an extent such that its osmotic pressure was similar to that of the external solution and the remainder of the cell was filled with the external solution. If, at the beginning of the determinations, the surrounding solution were of lower concentration than the osmotic pressure of the cell sap, continued swelling would be expected until the DPD of the cells balanced that of the external solution.

These phenomena could be expected to result in the situation as portrayed in the diagram, where it can be seen that at DPD values in excess of the plasmolytic value of the cell sap (represented by moderate and severe wilt), tissue weight and volume increased in each of the floating treatments. The fact that an estimate was obtained for tomato at moderate wilt was possibly due either to the fact that the DPD was not quite at the plasmolytic value, or that the cell walls in tomato were less rigid than those of privet and tended to collapse to some extent in plasmolysing solutions, resulting in some loss of weight and volume.

In the determinations in which estimates of DPD were obtained by the floating methods, the values were always higher than those obtained by the vapour method. This could be attributed to the fact that, in any one tissue, cells with a range of osmotic characteristics exist and those with sap of low concentration would be plasmolysed by solutions in which most of the other cells would still retain some turgor. As further change in the dimensions of a cell virtually ceases with onset of plasmolysis, this would result in the final dimensions or weight of the tissue being greater in the floating treatments than in vapour and would, as a result, tend to progressively overestimate DPD as the number of plasmolysed cells increased.

These various explanations were supported by a demonstration in which turgid disks of tomato and privet leaf tissues were dehydrated, some by being floated in solutions of known osmotic potential and others by being placed in vapour of known water potential. The results are given in Figure 7. The dehydration periods were 2 hr for the floating treatments and 32 hr for the vapour treatments. Because the use of NaCl as a plasmolysing agent might be questioned due to its tendency to infiltrate into the vacuole in the floating treatments, data are presented for both NaCl and mannitol, the latter being widely accepted for this purpose. The highest concentrations of mannitol it was possible to maintain were of 25 atm osmotic potential.

From the diagram it can be seen that as the DPD approached the value at incipient plasmolysis (estimated from cryoscopic determinations as 11·5 atm for tomato and 21·5 atm for privet) the two curves diverged, and that in the case of the floating disks no further decrease, in fact a slight increase, was evident after this point was reached. These features support the explantion, provided above, of progressive plasmolysis of the cells of the tissue with increasing concentration of the surrounding solution, and confirm that change in cell size is slight after plasmolysis. The slight increase in weight in the solutions of highest concentration, if significant,

could be explained on the basis of infiltration of the external solution into the vacuole, which could cause an increase in the osmotic pressure of the cell sap and an associated slight increase in cell volume at incipient plasmolysis. These results would appear to confirm that floating methods can only be expected to operate at DPD's up to those at incipient plasmolysis, and that errors will begin to be introduced as soon as some cells of the tissue reach this value.
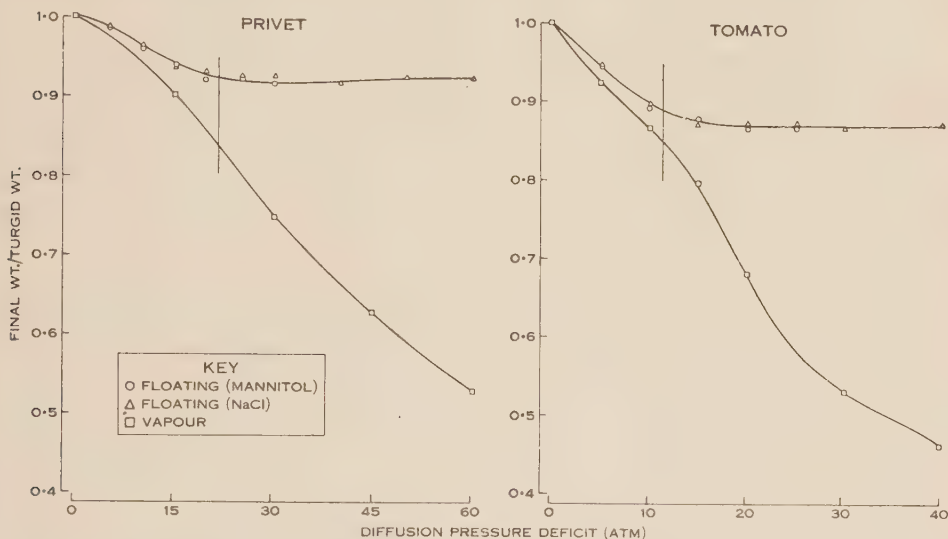


Fig. 7.—Dehydration of turgid disks of privet and tomato leaf tissue in water vapour of different water potential and in graded solutions of mannitol and NaCl.

## IV. Discussion

The primary factors influencing the accuracy of the vapour method for measuring DPD appear to be the difficulty of obtaining steady equilibrium values and the marked differences in rates of sorption and desorption.

The data of Figures 2 and 3 demonstrate that the rate of water exchange between the disks and the surrounding vapour shows a characteristic approach to an equilibrium condition, rate of water exchange decreasing as the DPD of the tissue approaches that of the vapour. Instead of reaching a steady value, however, continued loss of weight occurs at a slow, but fairly steady rate. This is particularly noticeable in the sorption curves of Figure 3, and is probably attributable to the effects of respiration. If so, it may continue at a changing rate throughout the determination, depending on the water content of the tissue at any one instant (Iljin 1957), and could introduce a potential source of error by causing changes in dry weight and hence in the osmotic characteristics of the tissue. However, experimental data shows that tissue refloated on water after dehydration regains almost exactly the same weight as the original turgid weight before dehydration. Since any significant change in the osmotic characteristics of the tissue would alter the volume of water in the tissue at full turgor, this suggests that any carbohydrate transformations which take place during dehydration are of small extent, or are

reversible, and that the dry weight losses caused by respiration do not unduly influence the osmotic characteristics.

The lack of attainment of stable equilibrium values means that an arbitrary time must be set for the determination. As DPD is estimated as being equivalent to the water potential of the vapour in which the tissue would neither gain nor lose weight, such a procedure in itself does not introduce errors, but the relatively slow rates of sorption compared to desorption tend to affect the estimate obtained.

The data of Figure 5 provide evidence as to the divergence of the sorption parts of three curves from an overall desorption curve and indicate that at the DPD's concerned, differences of up to 5 atm were obtained between estimates made from the desorption curve and those from the standard curve. Although this difference appeared to be primarily due to slow rates of sorption, it is possible that incomplete desorption may also have been of influence and both these factors could have been complicated by hysteresis effects.

Although it has not been possible to obtain direct evidence of hysteresis in these experiments, hysteresis is a property of porous and colloidal materials and has been identified in living and dead plant material (Rao, Rao, and Rao 1949; Kelsey 1957). In both instances cited, the divergence of the sorption and desorption parts of the hysteresis loop amounted to a difference of up to 2–3 per cent. water content, at relative vapour pressures equivalent to the DPD's used in this paper. In the experiments presented in Figure 5 a difference in water content of this magnitude would represent a DPD difference of up to 5 atm, a value sufficient to explain the discrepancy observed. While this deduction is speculative, it must be recognized that hysteresis is almost certainly of influence in causing differences between sorption and desorption curves.

Whether or not hysteresis is of influence, it is apparent from Figure 5 that the standard method for measuring DPD will always tend to provide an underestimate due to the relatively slow rates of sorption. This feature appears to be extremely difficult to eliminate. A possible alternative is to use the desorption curve alone, as in Figure 5. Such a procedure has the advantage of considering only the sorption part of the curve, and it also enables the simultaneous measurement of relative turgidity* and DPD and the development, if desired, of relative turgidity/DPD relationships for the tissue under study (Weatherley and Slatyer 1957). However, this method also has several disadvantages. These are primarily associated with the need to obtain final equilibrium values in order to obtain an accurate estimate of DPD. As has been mentioned above, such values were not obtained in this series of experiments, although the final stage was reached in most of the desorption curves in about 32 hr. If the final stage does not represent an equilibrium condition an overestimate of DPD would be obtained. Furthermore, if hysteresis is of significance, as seems probable, an estimation of DPD made on the desorption curve would only apply if the plant as a whole were in a desorption phase when sampled. Although this no doubt applies during the period of increasing transpiration each morning,

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*"Relative turgidity" has been defined by Weatherley (1950) as the ratio of the amount of water present in a quantity of tissue when sampled compared with the amount present in the same quantity of tissue when fully turgid.

it is probable that for most of the day the plant, under normal conditions, is in a sorption phase.

For these reasons, it seems preferable to use the standard method for DPD measurement, except where it is especially desired to establish relative turgidity/DPD relationships for different plant tissues. The extent of the error involved in determinations with the standard method will vary with the relative rates of sorption and desorption and with the possible influence of hysteresis. If hysteresis occurs to the extent observed by Rao, Rao, and Rao (1949), the divergence between the sorption and desorption portions of the hysteresis loop could vary from zero at zero DPD to as much as $\pm$ 3 atm at the highest DPD's employed. Although this order of variation is high, it would not be confined to vapour determinations, but would apply to any determinations of DPD in which sorption and desorption processes are involved.

The standard method therefore tends to provide an estimate of DPD midway between the sorption and desorption curves, although as mentioned above the relatively slow rates of sorption will normally cause an underestimate. In practice, the values obtained, by comparison with estimates from floating methods, have been found to lie within a range not greater than $-3$ to $+1$ atm about the true value. This error can be reduced further by using a series of constant-humidity chambers, each of which differ by only 2–3 atm water potential from the next, so that the sorption and desorption parts of the curve can be well defined and any misleading value identified.

The comparative study between the standard floating methods for measuring DPD and the vapour method was of considerable value in determining the reasons for the decreasing effectiveness of the former methods as the DPD approached the value at incipient plasmolysis. The explanation provided, that when plasmolysis occurs in any one cell change in weight or volume of the cell virtually ceases, appears to be logical and satisfactory. In any one piece of tissue, cells with a range of osmotic characteristics exist, and as these progressively become plasmolysed the sensitivity of the floating methods can be expected to decrease until the whole tissue becomes plasmolysed, when the methods are virtually ineffective. It could be expected that in cells which did not readily plasmolyse and in which the walls collapsed readily with contraction of the cytoplasm, some continued loss of weight and volume might occur. No evidence of this was observed in the present experiments. On the other hand, the slight increase in weight and volume observed in some instances, where tissue was in hypertonic solutions, suggested that the external solution was to some extent entering the vacuole, raising the osmotic potential of the vacuolar sap, and hence raising the volume and weight of the tissue at incipient plasmolysis.

Despite the fact that these methods were insensitive at DPD's greater than at the plasmolytic value, at lower DPD's they gave good agreement with the vapour method, and at DPD's of only a few atmospheres, where the floating methods are most sensitive, it is probable that they are of greater accuracy.

A final point in connection with the data of Figure 7 is that the gradual divergence of the dehydration curves obtained in vapour and in solution demonstrates that the "minimum cell volume" method of measuring osmotic pressure (Ursprung

1923) could be subject to important errors. This method contends that osmotic pressure of the cell sap is isotonic with the most dilute solution in which a segment of tissue can be dehydrated to its minimum volume (or some other dimension). It depends on the curve of decreasing volume against increasing concentration having a sharp flex point from which the critical concentration can be estimated. The data in Figure 7 indicate that not only would the gradual shrinkage of the tissue prevent a sharp flex point being obtained, but also that the progressive plasmolysis and infiltration of the cells would tend to shift the point at which the minimum value was reached, increasing the value obtained.

## V. Acknowledgments

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# NODULATION STUDIES IN LEGUMES

## I. THE SYNCHRONIZATION OF HOST AND SYMBIOTIC DEVELOPMENT IN THE FIELD PEA, PISUM ARVENSE L.

### By J. S. Pate*

[*Manuscript received December* 20, 1957]

### Summary

Symbiotic development of two varieties of field pea (*Pisum arvense* L.) was studied by periodic sampling from populations of field-grown material. Various features of effective symbiosis were found to be characteristically synchronized with host plant life cycles.

Nodule initiation was complete at the 7-leaf stage of plant development. Peak values in total nodule numbers and nodule/plant weight ratio occurred at this stage. Later, the regular decline in nodule numbers was offset by large increases in the size and fixation efficiency of remaining nodules.

Well-defined pigment changes occurred in nodule populations. Numbers of young (white) nodules developed during precocious root expansion in the 1–3-leaf stages. Haemoglobin formation was rapid in all nodules. The first-developed nodules on primary roots were pigmented (red) just before cotyledon nitrogen reserves were exhausted. Maxima in total red nodules in both varieties were attained in the 6–8-leaf stages. The subsequent active (red) life of nodules varied from 8 to 80 days. Senescent (green) nodules accumulated on roots following extensive haemoglobin destruction in the nodules of maturing plants.

Average nodule fresh weight increased some 30–50-fold during plant growth. Comparisons of the average sizes of green and red nodule-population samples revealed a progressive elimination of smaller nodules throughout plant development.

Nodule nitrogen and haemoglobin concentrations increased together to peak values in mid-vegetative stages. The maximum in total red-nodule nitrogen which occurred just before flowering, and some 7-leaf units before a maximum in total plant nitrogen, reflected a general increase in nodule fixation efficiency as host plants matured.

Approximately 30 per cent. of the nitrogen in the red nodule was removed as nodules turned green in early senility. Nitrogen returns from this source were estimated at less than 3 per cent. of the fixation benefit from healthy (red) nodule activity. High nodule–plant nitrogen transfer rates were recorded from red nodules from the 5-leaf stage until plant flowering.

## I. Introduction

Early observations of symbiotic nitrogen fixation in the annual legume led to the conclusion that legumes were rich in nitrogen at maturity through concerted nodule utilization in flowering and fruiting stages of host plant development. It was tacitly assumed that fixed nitrogen was stored in nodules and later released in host digestion of aging bacterial tissues (see monograph by Fred, Baldwin, and McCoy 1932).

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G. Bond (1936) was prominent among the first authors to question any substantial host plant benefit through nodule emptying. In the soybean plant he described a regular and immediate transfer of fixation products from nodule to host plant in stages of plant growth where there were no signs of any degenerative changes in nodule populations.

Wilson and Umbreit (1937) recognized three important phases in soybean symbiosis. First, a short initial phase when a large proportion of fixed nitrogen was retained in the nodules. Then, a longer phase, extending over most of vegetative growth, when a large-scale transfer of fixation products took place giving logarithmic increases in plant growth and nitrogen content. Finally, a phase when fixation slowed down, nodule to plant transfer was highest, and nodule to plant weight ratios decreased.

Further studies by the Wisconsin school established a close interdependence of symbiotic nitrogen fixation and carbon assimilation processes in legume development (see Wilson 1940).

The discovery of nodule haemoglobin as an integral part of the fixation mechanism has led to considerable expansion of earlier concepts of symbiosis. Virtanen and co-workers found a positive correlation between nitrogen fixation rates and nodule haemoglobin concentrations in a number of legume associations. For the pea plant Virtanen *et al.* (1947) and Virtanen, Erkama, and Linkola (1947) showed that nodule haemoglobin development paralleled the course of fixation; that the commencement of fixation was attendant upon pigment formation in young nodules; and that the cessation of nodule fixation activity in fruiting plants was associated with widespread pigment destruction in senescent nodules. This relationship between pigment changes and nodule fixation activity has been noted for various legumes by other authors, e.g. Jordan and Garrard (1951), Nowotny-Mieczynska (1952), and Heumann (1952).

The aim of this series of investigations is to provide further information on the general pattern of symbiotic development in legumes. The present paper on nodulation of the field pea outlines various aspects of the initiation and functional life of root nodule populations in an effort to determine the role of the individual nodule in plant nitrogen economy. Further papers will attempt to evaluate the much neglected topics of environment and host determination of legume nodule activity and longevity.

## II. Materials and Methods

### (a) *Plant Material*

Symbiotic development in the field pea was studied by periodic sampling from two series of sowings:

1954 *Series.*—*Pisum arvense* L., var. New Zealand maple pea—summer sowing, growing period June 11, 1954, to August 30, 1954.

1955 *Series.*—*Pisum arvense* L., var. Black-eyed Susan—summer sowing, growing period May 25, 1955, to August 28, 1955.

Both varieties were grown in 60 by 40-ft plots of red subsoil sand at Cherryvalley, Belfast, Northern Ireland. The ground was chosen for its light texture and

low plant nutrient content. A balanced nitrogen-free mineral fertilizer applied to the plots before each sowing created conditions suitable for maximum symbiotic development. Row cultivation was practised to facilitate extraction of nodulated roots. Approximately 10,000 seeds were sown in each season to give plant populations well in excess of predicted numerical demands in sampling.

A seed-applied rhizobial supplement was given to each series since the subsoil of the plots was found to be markedly deficient in indigenous *Pisum*-group rhizobia. Inoculants were derived from a stock culture originally isolated from an effective nodule of maple pea. A satisfactory set of effective nodules was obtained in both seasons allowing for uniform symbiotic development in both varieties.

### (b) Sampling Procedure

Fifty plant samples were randomly selected from the plots at 3–7-day intervals in the life cycles of the host plants. This rather large sample unit was necessary for the construction of reliable nodulation histories from soil-grown legume sowings, and involved a $0 \cdot 5$–5 per cent. standard error in host or symbiotic characters.

Extreme care was exercised in the lifting and washing of plant root systems. Diseased, chlorotic, or otherwise atypical plants were rejected from samples.

### (c) Recordings from Samples

The following quantities were recorded for each sample:

(i) *Age.*—Age of the sample in days from sowing.

(ii) *Developmental Age (expressed as average number of expanded leaves per plant).*—Leaf age is of special value in gauging progress towards maturity in different seasons of growth.

(iii) *Nodule Number.*—All nodules visible to the naked eye were recorded in population counts on primary and secondary root systems of the plants of the sample.

(iv) *Nodule Colour.*—Three colour classes were recognized in nodule counts:

*White* (W-*type*) *Nodules.*—Small young nodules with cream-coloured contents, marking sites of recent infections on root systems.

*Red* (R-*type*) *Nodules.*—Bacteroid-filled nodules with haemoglobin-pigmented bacterial tissues.

*Green* (G-*type*) *Nodules.*—Senescent nodules showing haemoglobin decomposition into bile-type pigment. Nodules remain green for a short period before entry by foreign bacteria causes extensive necrosis of internal tissues. A nodule was classed as G-type once more than two-thirds of its proximal bacterial tissues had turned green. Brown, disintegrating nodules were ignored in all nodule counts.

(v) *Nodule Weight and Tissue Analyses.*—Nodules were removed by a razor stroke parallel to and touching the root surface. Representatives of the $W+R$ and the $G$ colour classes were separated, and both of these colour samples were weighed and subjected to Kjeldahl digestion. Determination of total nitrogen from aliquots of nodule or host plant tissue followed the conventional semi-micro-

Kjeldahl method, using a Markham steam-distillation outfit for ammonia recovery from digests. Portions of the 1954 series $W + R$ samples were analysed for nodule haemoglobin. Nodule haemoglobin was extracted as pyridine haemochromogen (see Virtanen *et al.* 1947; Virtanen, Erkama, and Linkola 1947) and the reduced haemochromogen estimated spectrophotometrically at 555m$\mu$. The latter absorption maximum gives least interference from the green pigment (see Hartree 1955).

(vi) *Host Plant Weight and Analysis.*—Average fresh weights of individual plant organs were determined for each sample. In the 1955 series, plant tops and roots, cotyledons, fruits, and flowers were subjected to Kjeldahl analysis for total nitrogen. Nitrogen analyses were confined to cotyledon and nodule tissues in the 1954 series.

## III. Results

Various aspects of the symbiotic development of the field pea are summarized in the figures and table accompanying the text.

### (a) Nodule Number (Figs. 1(a), 1(b))

Extensive and rapid infections of primary and secondary root systems result in sharp peaks in total nodule numbers in both varieties in the 6–8-leaf stages. A subsequent regular decline in nodule numbers occurs in late vegetative and reproductive development. In the 1954 series some 20–40 per cent. of nodule populations persisted on roots until the end of the plant life cycle. The 1955 season allowed a more normal ripening of fruits, and this series recorded a higher efficiency of nodule utilization.

The two varieties show marked differences in primary root, but not in secondary root, nodule numbers. In other trial sowings it was found that primary root nodule intensities in a legume species varied considerably with soil and prevalent light and temperature conditions, and consequently the observed differences are not taken to represent varietal differences in rhizobial acceptance. An analysis of this aspect of symbiosis in various members of the *Pisum* cross-inoculation group will form the basis of a further paper in this series.

### (b) Nodule Colour (Figs. 2(a), 2(b); 3(a), 3(b))

The four graphs depicting fluctuations in the numbers of $W$, $R$, and $G$ nodules over the host cycle provide conclusive evidence of ordered pigmentation sequences in nodule populations.

(i) *Root Infection* (W curves).—Nodule initiation on a particular root system is related to the preceding growth activity of that system, since infection in both varieties was consistently limited to root hair-invested portions of the roots. The early peaks in total $W$ nodule numbers may be correlated with precocious root expansion over the 1–3-leaf stages. A similar relationship between root growth and nodule inception has been noted by the author in annual species of *Lupinus*, *Lathyrus*, *Vicia*, *Trifolium*, *Medicago*, *Lotus*, and *Ornithopus*. These species all exhibited root hair-invasion patterns, and showed remarkably early development of the nodule-bearing parts of their primary and secondary root systems.

(ii) *Nodule Activity* (R *curves*).—Pigmentation of nodules follows a strict aging sequence on all root systems, where *W*-type nodules are consistently nearer root apices than their older, pigmented companions. Thus, inspection of the ascending portions of *W* and *R* curves gives a fairly reliable picture of pigmentation rates on the various root systems. A time lapse of 1–6 days occurs between a nodule being first observed on a root system and its being recorded as showing visible
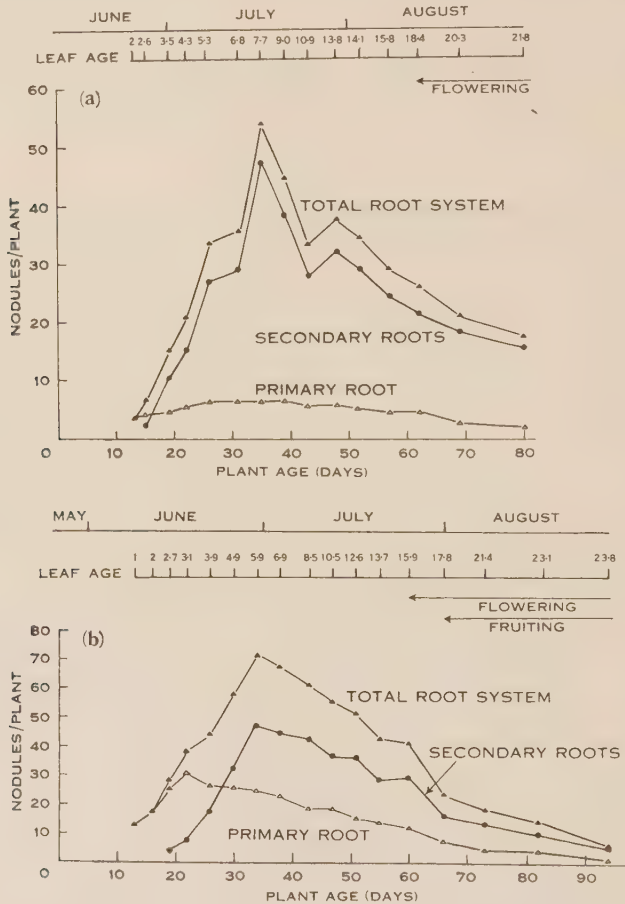


Fig. 1.—*P. arvense*—nodulation: nodule numbers on primary, secondary, and complete root systems. (*a*) 1954 series, var. New Zealand maple pea; (*b*) 1955 series, var. Black-eyed Susan.

haemoglobin colour. In both series first-formed nodules on a root system are slower to develop pigment than those arising from later infections. This is thought to be largely a seasonal effect since, in comparisons of pigmentation rates in *Vicia sativa* L. as a winter and as a summer annual, later-formed nodules on a root system coloured more slowly than their older companions in autumnal conditions, but more quickly in spring conditions (Pate, unpublished data).

(iii) *Nodule Senescence* (G *curves*).—The active life of nodules in the sets of effective nodules of the two series is of very variable duration, healthy haemoglobin-pigmented nodules remaining on a root system for anything from 8 to 80 days. The total root system maxima in G nodule numbers are attained at plant flowering. It is to be expected that senescent nodules will accumulate in late vegetative development.
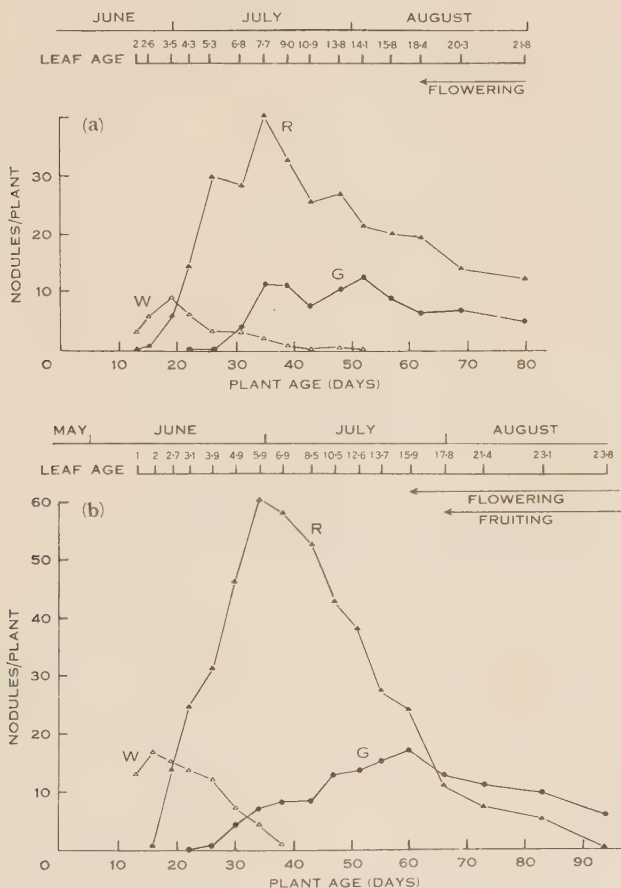


Fig. 2.—*P. arvense*—nodulation: nodule colour, complete root system. *W*, young white nodules; *R*, red actively fixing nodules; *G*, green senescent nodules. (*a*) 1954 series; (*b*) 1955 series.

(*c*) *Nitrogen Levels in Plant Tissues* (*Figs.* 4(*a*), 4(*b*))

Nitrogen concentrations in plant parts are expressed on a fresh weight basis.

(i) *Nodules.*—As the nodule population becomes increasingly active in fixation nitrogen levels in the $W+R$ samples rise. A significant drop in nodule nitrogen level is noted in mid-vegetative growth for the 1954 series, but not for the 1955 series. A post-flowering decrease in red-nodule nitrogen levels is noted in both varieties.

Parallel increases in concentrations of nodule haemoglobin and nodule nitrogen are noted for the $W+R$ sample of the 1954 series. Maxima in both quantities are attained early in host development.
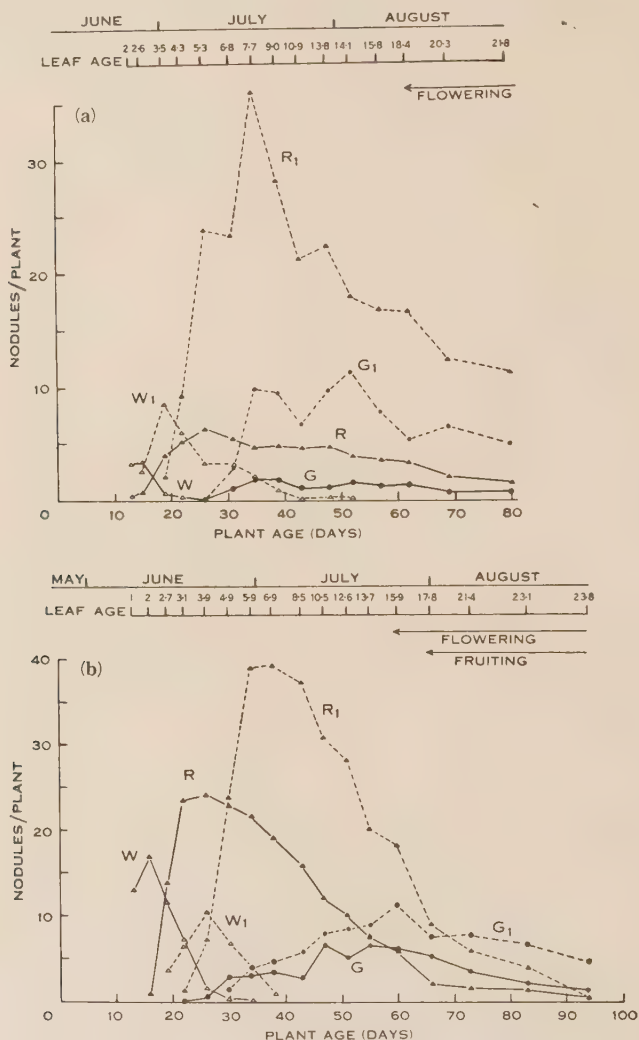


Fig. 3.—*P. arvense*—nodulation: nodule colour, primary and secondary root systems. $W$, $R$, and $G$ symbols as in Figure 2. $W$, $R$, and $G$ curves: primary root system; $W_1$, $R_1$, $G_1$ curves: secondary root system. (*a*) 1954 series; (*b*) 1955 series.

$G$-nodule nitrogen concentrations are about 60–80 per cent. of current values for $W+R$ nodules. As the nodule does not change in shape or turgidity while pigment destruction is proceeding it can be assumed that approximately one-third of the total nitrogen in the nodule is removed in early senility.

(ii) *Host Plant Tissues.*—Cotyledon nitrogen levels decrease as nitrogen is removed in seedling growth. The minimum value for plant nitrogen concentration coincides with the transition period between cotyledon and nodule nitrogen nutrition. Nitrogen levels rise to a pre-flowering maximum associated with the initiation of flowering primordia.
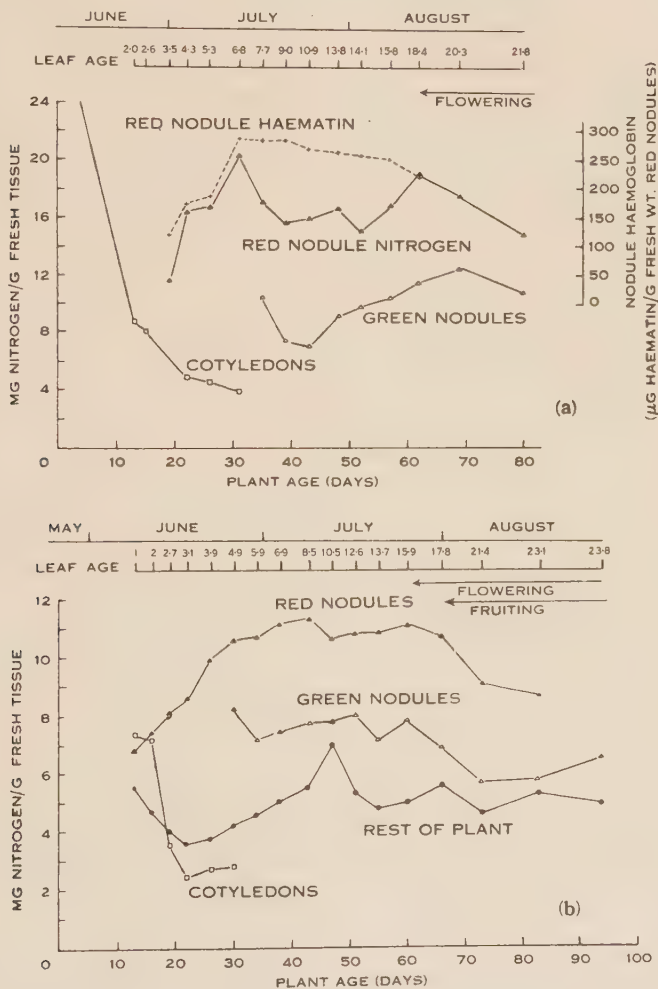


Fig. 4.—*P. arvense*—nodulation: nitrogen concentrations in nodule and host plant tissues. (*a*) 1954 series (also with red-nodule haemoglobin levels); (*b*) 1955 series.

(d) *Nitrogen Content of Plant Tissues, 1955 Series (Figs. 5(a), 5(b))*

Figure 5(*a*) outlines the nitrogen economy of the young seedling. The cotyledon source is depleted towards the end of the 3-leaf stage. A small residue of unavailable nitrogen, amounting to less than 20 per cent. of the initial seed reserves, is lost as cotyledons are sloughed off the seedling axis.

Total plant nitrogen remains fairly constant over early seedling stages, but rises sharply once nodules turn red and commence fixation. There is no evidence of a check in plant development through a nitrogen hunger period following exhaustion of cotyledon reserves.
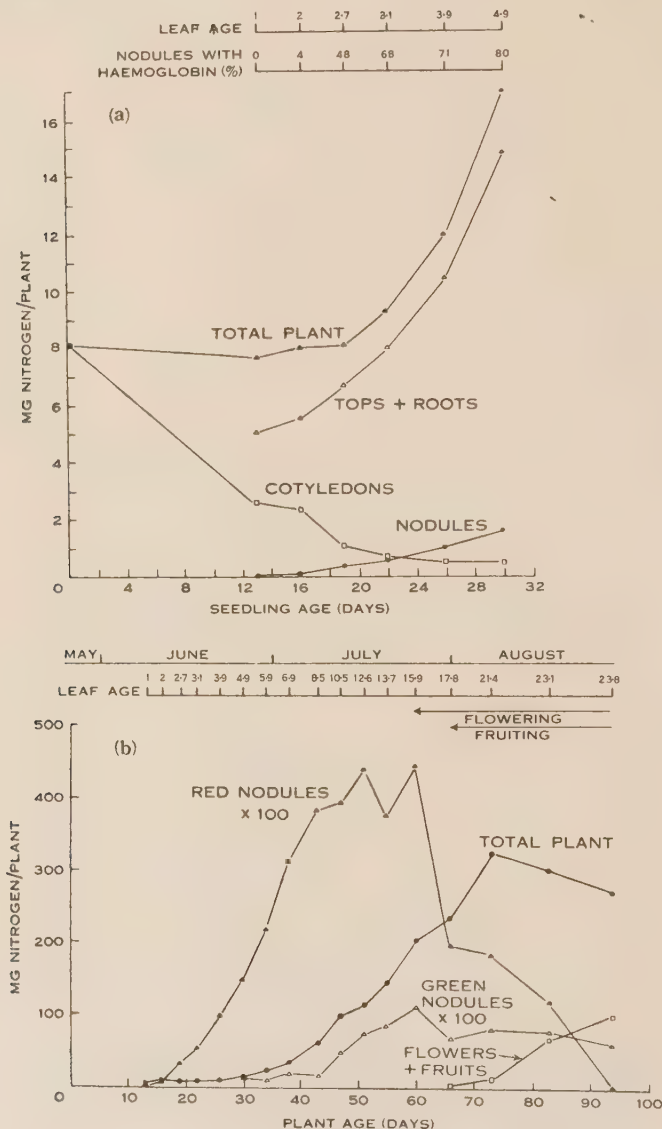


Fig. 5.—*P. arvense*—nodulation: nitrogen content of nodule and host plant tissues (1955 series): (*a*) seedling nitrogen economy; (*b*) nitrogen contents of host plant and nodules over the complete life cycle.

Plant red-nodule nitrogen increases rapidly and shows a well-marked peak slightly before flowering. Green-nodule nitrogen represents a much smaller proportion

of total plant nitrogen.  The significance of nitrogen withdrawal in nodule aging is considered in Section IV.

Total plant nitrogen increases in a sigmoid fashion with a maximum in late flowering stages some 2–3 weeks (7-leaf units) later than the nodule nitrogen maximum.  This lag between nitrogen accumulation in symbiotic organs and in the complete plant can be explained on the assumption that nodules become more efficient in fixation as they mature.

### (e) *Average Nodule Size (Fig. 6)*

Remarkably similar sets of curves are obtained for the two series:

W + R *Sample.*—Average nodule size (fresh weight) increases slowly in early plant growth since new nodules are being constantly added to plant root systems.
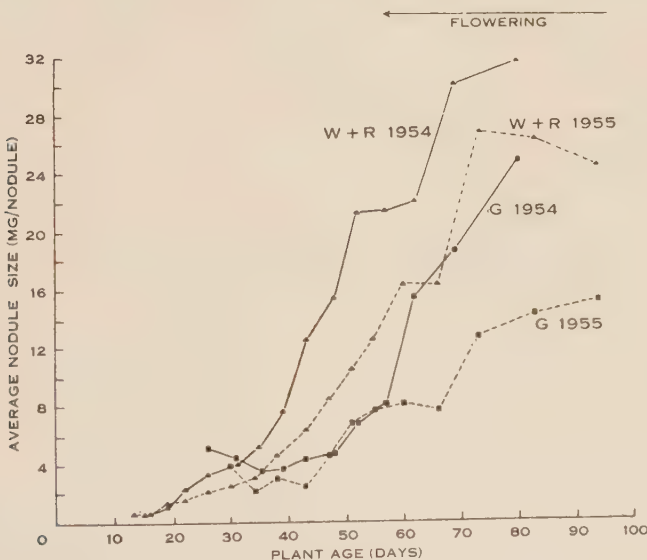


Fig. 6.—*P. arvense*—nodulation: average nodule size.  $W+R$, young white + red actively fixing sample; $G$, senescent green nodule sample.  1954 series, var. New Zealand maple pea; 1955 series, var. Black-eyed Susan.

Once nodule initiation ceases existing nodules grow rapidly, average size being increased some 30–50-fold in later plant growth.  Nodules may show active meristematic apices almost until the death of the adult plant.  Mature nodules are of smaller average size in the variety Black-eyed Susan.

G *Sample.*—The first samples of green nodules are of greater average size than their active contemporaries since they represent the more mature portions of a population composed of many small recently developed nodules.  At all stages of later development green nodules are of much smaller size than members of the current red-nodule population.  This must be interpreted as a progressive elimination of the smaller nodules on root systems throughout host plant development.

### (f) Nodules Weight/Total Plant Weight Ratio (Fig. 7)

This ratio may be considered as an index of the relative significance of the symbiotic organs at a particular stage in the plant life cycle. The ratio is low in early vegetative growth, rising to a peak value of 4–5 per cent. of the plant weight during the 4–6-leaf stages in both varieties. The ratio is progressively lowered in later development, until at plant maturity less than 0·5 per cent. of the total plant fresh weight is comprised of nodule tissue.

### IV. Discussion

Figure 8 summarizes the relationship between host plant development (as gauged by leaf production and nitrogen accumulation) and the various representations of symbiosis studied. Remarkably similar sequences of attainment of symbiotic maxima are recorded for the two varieties. The Black-eyed Susan variety
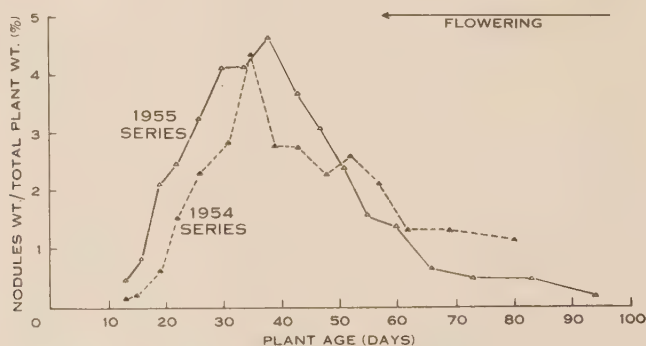


Fig. 7.—*P. arvense*—nodulation: fluctuations in the ratio nodules weight/total plant weight, 1954 and 1955 series.

flowers some two leaf units earlier than New Zealand maple pea and shows comparable earliness in symbiotic development on a leaf age basis. It would be interesting to know whether early-flowering varieties show more precocious nodulation sequences than late-flowering varieties of the same species. Similarly, would photoperiodic or vernalization induction of earlier flowering be anticipated by a general hastening of symbiotic development in earlier stages of vegetative development?

Each feature of symbiosis studied adds its own information to the general pattern of integration of host and rhizobial activities, and it is possible to construct the following phasic analysis of symbiotic development for the field pea (see also Fig. 8).

### (a) Phase I—Seedling Growth (1–3-leaf stages)

Cotyledon reserves are the major nitrogenous source in this phase. The essential nodule-bearing portions of the root system are expanded, and large numbers of young nodules accumulate on roots. The ordered infection sequences on primary and secondary roots offer evidence of root hair-invasion patterns in the field pea,

as have been recorded for other members of the subtribe Vicieae (L. Bond 1948; Nutman 1956).

In the middle of the seedling phase the first-formed nodules of the primary root become haemoglobin-pigmented. The end of the phase shows a minimum in plant nitrogen concentration. In fixation studies of *Vigna* and *Cicer* in Indian conditions, Raju (1939) described critical lag periods between sowing and nodulation, and between nodule formation and subsequent fixation. These periods were found to be more prolonged and accentuated in poor light conditions or inefficient
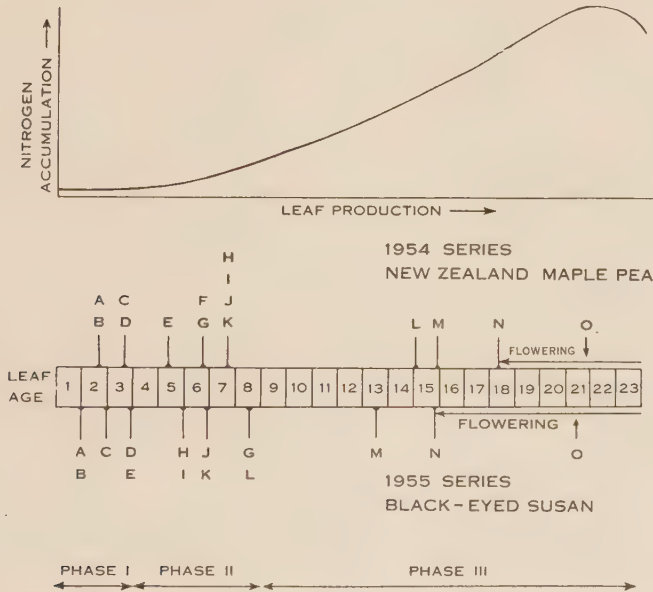


Fig. 8.—Diagram summarizing the synchronization of host and symbiotic development in two varieties of field pea, *P. arvense*. Various representations of the nodulation cycle are related to host plant leaf age and nitrogen accumulation. *A*, maximum primary-root white nodules; *B*, first nodule turns red; *C*, 90 per cent. of cotyledon nitrogen exhausted; *D*, maximum secondary-roots white nodules; *E*, maximum primary-root red nodules; *F*, maximum haemoglobin concentrations in red nodules (1954 series); *G*, maximum nitrogen concentration in red nodules; *H*, maximum total-roots nodules; *I*, maximum total-roots red nodules; *J*, maximum nodules/plant weight ratio; *K*, maximum secondary-roots red nodules; *L*, nodule initiation ceases; *M*, 50 per cent. of red nodules destroyed; *N*, maximum in red-nodule nitrogen/plant; *O*, maximum in total plant nitrogen.

symbiosis. There is no evidence here of any such nitrogen hunger period in the transition period between cotyledon and nodule nitrogen nutrition despite the fact that the varieties were grown in soil abnormally low in combined forms of nitrogen. This would question the real value of the local Irish practice of giving nitrate supplements to legumes at sowing. Although added inorganic nitrogen might benefit seedling growth it might endanger subsequent fixation by suppressing nodule development. Further work is obviously required on this important aspect of nodulation.

### (b) *Phase II—Early Vegetative Growth* (*3–8-leaf stages*)

This phase of development witnesses maxima in total nodule numbers, in total red nodules, and also in the ratio nodule weight/plant weight. Peak values in red-nodule haemoglobin and nitrogen concentration also occur, fixation efficiency increases, and nodule to plant transfer ratio increases. Pigmentation of nodules is completed, and some green nodules are recorded on roots.

### (c) *Phase III —Late Vegetative and Reproductive Development* (*8–23-leaf stages*)

There is a regular decline in nodule numbers over phase III resulting in the loss of some 60 per cent. of red nodules by the commencement of flowering. This early destruction of nodule populations in the field pea is particularly interesting. Companion studies on vetch (*Vicia sativa* L.) nodulation showed a much later maximum in nodule numbers, with extensive nodule shedding delayed until flowering had commenced. Fred, Baldwin, and McCoy (1932) have recorded a similar nodule emptying in fruiting stages of large-seeded legumes. The early peak in nodule numbers in the field pea is undoubtedly associated with a completion of root development well before plant flowering. However, this fails to account for the premature nodule *aging* noted in both series. In mixed nodule populations derived from several rhizobial forms one might expect an early loss of less-effective symbiotic units. Here a very variable active life of 6–80 days is recorded from the symbiotic performance of one effective *Rhizobium*. Possibly some host factor induces early nodule destruction. There is considerable evidence of internodular inhibitions in nodule initiation (see Nutman 1956) and similar interactions may obtain later in nodule life. Alternatively, competition for some nutrient factor, e.g. carbohydrate, might account for wide variations in nodule life span. This latter view is consistent with the observation that smaller nodules are progressively shed from roots at all stages of development.

The pattern of nitrogen accumulation over phases II and III may be related to fixation activities of nodule populations in the following manner:

(i) *The Fixation Potential of the Nodule Population.*—Plant red-nodule nitrogen is probably the most adequate expression of the fixation potential of the plant in that it is obviously related to the volume of active bacterial tissues (see Fig. 5(*b*)). Both G. Bond (1936) and Aprison and Burris (1952) describe fixation rates in terms of the nitrogenous contents of the nodule.

(ii) *The Apparent Fixation Efficiency of Red Nodules.*—Fluctuations in apparent fixation intensity in red nodules are recorded in Table 1 (1955 series). Fixation rates are calculated as milligrams nitrogen fixed/gram red nodules/day, assuming that all nitrogen accumulated above cotyledon level is derived from nodule activity, and that there is no wide-scale excretion of fixation products from nodules to external medium. The validity of either assumption was not checked, but it is unlikely that excretion would occur in the rapid growth conditions in both seasons, and it was known that the subsoil of the plots was extremely low in combined forms of nitrogen.

Significant fixation activity is recorded from the 3-leaf stage until the beginning of fruiting, and shows a general increase trend of from 7 to 65 mg nitrogen fixed/g red nodules/day. The extremely high nodule activity on flowering plants accounts for the lag between maxima in weight of red nodules and total plant weight. There is some evidence of a final burst of activity prior to nodule destruction, but the nodule population at this stage is composed of large mature nodules with relatively

TABLE 1

THE RELATIONSHIP BETWEEN NODULE FIXATION ACTIVITY AND NITROGEN ACCUMULATION IN
THE FIELD PEA, PISUM ARVENSE L. (1955 SEASON)

| Plant Leaf Age (stages) | Increment in Total Plant Nitrogen (mg) | Apparent Fixation Intensity in Red Nodules (mg N fixed/day/g red nodules) | Nodule to Plant Transfer* | Nitrogen (mg) Released in Nodule Decomposition $(N)$† | Nitrogen (mg) Available through Reduction in $R_N$† | Retention of Fixed Nitrogen (mg) in Persisting Nodules‡ |
|---|---|---|---|---|---|---|
| 2·7– 3·1 | 1·2 | 7·2 | 81·6 | — | — | 0·21 |
| 3·1– 3·9 | 2·7 | 8·2 | 83·6 | — | — | 0·43 |
| 3·9– 4·9 | 5·0 | 10·5 | 90·0 | ⎫ | — | 0·72 |
| 4·9– 5·9 | 8·1 | 11·7 | 91·5 | ⎬ 0·64§ | — | 0·90 |
| 5·9– 6·9 | 10·4 | 10·7 | 90·1 | ⎭ | — | 1·18 |
| 6·9– 8·5 | 27·0 | 17·3 | 97·4 | 0·17 | — | 0·86 |
| 8·5–10·5 | 37·4 | 26·4 | 99·7 | 0·40 | — | 0·50 |
| 10·5–12·6 | 14·9 | 9·7 | 96·9 | 0·22 | — | 0·68 |
| 12·6–13·7 | 31·6 | 20·9 | 99·9 | 0·86 | — | ⎫ |
| 13·7–15·9 | 57·0 | 30·4 | 100+ | 0·21 | — | ⎬ 1·57 |
| 15·9–17·8 | 32·7 | 18·5 | 100+ | 1·17 | ⎫ | — |
| 17·8–21·4 | 87·1 | 65·0 | 100+ | 0·37 | ⎬ 2·42 | — |
| 21·4–23·1 | — | — | 100+ | 0·27 | ⎬ | — |
| 23·1–23·8 | — | — | 100+ | 0·61 | ⎭ | — |
| Totals | 315·1 | | | 4·92 | 2·42 | 7·05 |

* Percentage increment in nitrogen in plants minus nodules/increment in total plant nitrogen.

† See text for explanation of symbols and method of calculating $N$.

‡ Expressed as red-nodule nitrogen increment plus nitrogen loss in nodule emptying.

§ Approximate value based on a loss of 20 nodules from roots over the 3·9–6·9 leaf stages (see Section IV).

larger volumes of bacterial tissues. Furthermore, overall efficiency may increase with advancing season, and with the progressive loss of the smaller, possibly less active, members of the nodule complement.

(iii) *Apparent Nodule to Plant Transfer.*—This is defined as percentage nitrogen increment in plant minus nodules/nitrogen increment in total plant. Values recorded in Table 1 show a progressive increase in transfer from 80 to 100 per cent. as nodule

populations mature. Similar ranges of values have been noted for soybean symbiosis by G. Bond (1936) and Wilson and Umbreit (1937).

(iv) *Nitrogen Available to Host Plant or Returned to the Soil in Nodule Decomposition.*—No data are available for annual legumes on this much-debated aspect of nodule nutrition. Butler and Bathurst (1957) recently provided an estimate of 72 lb nitrogen/acre/year available through nodule decomposition in New Zealand perennial clover and ryegrass swards. Their estimate corresponds to an annual release of 240 mg of nitrogen from a single white clover plant.

The data on nodule-colour changes in the 1955 series enable a fairly accurate estimate to be made of nitrogen increments to plant or soil in nodule aging. Over the 3–7-leaf stages, nodule turnover is difficult to assess as nodules are still being added to roots. Later, once nodule initiation ceases, nitrogen available from nodule shedding ($N$) can be calculated from the expression:

$$N = R_n \times R_N \times G_W \,,$$

where

$R_n$ = number of red nodules lost over a particular period,

$R_N$ = current nitrogen concentration in red nodules, and

$G_W$ = current average weight of green nodules (the weight of the smaller members of red-nodule populations being eliminated from roots).

Values for the quantity $N$ over the age increments studied are included in Table 1. Total nitrogen available in nodule decomposition is found to be $4 \cdot 92$ mg/plant/season. The amount of this actually sequestered by the host plant is problematical. Even on the assumption that there is a 100 per cent. efficiency of nodule emptying, plant benefit over the life cycle from this source would be only $1 \cdot 6$ per cent. of the fixation returns from healthy nodules. A further source of plant nitrogen in nodule aging is through a progressive lowering of red-nodule nitrogen concentration in fruiting phases. This is calculated as $2 \cdot 42$ mg/plant/season, giving a total release in nodule senescence of $7 \cdot 34$ mg nitrogen/plant/season. The latter is equivalent to $2 \cdot 4$ per cent. of fixation returns from active nodules.

The above data show that little benefit to associated crops could materialize from nitrogen returns to the soil in nodule emptying. Moreover, the extremely small quantities of nitrogen available from this source would be returned to the soil late in crop growth. Claims of nitrogenous benefit to companion cereals have been made for mixed sowings of maple pea and oats in Northern Ireland, and it is obvious that nitrogen transfer is from some other source than nodule disintegration. Decay of root tissues and active excretion of nitrogen from nodules are other possible sources of nitrogen benefit to a non-legume. If substantial benefit occurs early in legume and cereal development, it would be reasonable to assume that excretion was the major exchange mechanism in underground transference of nitrogen.

(v) *Retention of Nitrogen in Persisting Nodules.*—A fraction of the nitrogen fixed is retained in persisting members of nodule populations allowing for the 30–50-fold increases in average nodule size observed over the nodulation cycle. The observed increment in red-nodule nitrogen over a specific period actually underestimates nitrogen retention as nodule nitrogen losses have occurred in nodule

destruction.    Hence values in Table 1 are derived from the expression red-nodule nitrogen increment + nitrogen loss in nodule destruction.    The values recorded show considerable retention of fixed nitrogen in persisting nodules from the 3-leaf stage right up to flowering. This period corresponds to stages of development recording rapid increases in average nodule size.

## V. Acknowledgments

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# THE ACCUMULATION OF RADIOACTIVE IODINE IN THE THYROIDS OF GRAZING ANIMALS SUBSEQUENT TO ATOMIC WEAPON TESTS

By H. R. Marston*

*Summary*

Radioactivity due to $^{131}$I was found in thyroid glands collected from sheep and cattle depastured on areas in various parts of Australia subsequent to atomic weapon tests conducted during 1956 at Monte Bello and at Maralinga. Thyroids from cattle were found to contain up to 830 m$\mu$c $^{131}$I (37 m$\mu$c $^{131}$I/g of tissue) and from sheep, up to 144 m$\mu$c $^{131}$I (70 m$\mu$c $^{131}$I/g of tissue). The uneven degree of contamination of pastures was emphasized by the fact that some of the highest concentrations of $^{131}$I were observed in glands collected from individuals of flocks and herds grazing on terrain 1500–2000 miles distant from the site of the explosions. As the tests proceeded, fluctuations in the $^{131}$I content of the thyroid glands of grazing stock indicated that many areas received repeated dressings of radioactive debris.

Contamination of Adelaide and its environs with fall-out from the third Maralinga explosion provided an opportunity to establish unequivocally that extremely little if any of the considerable amount of $^{131}$I that became concentrated in the thyroids found its way to the glands via the lungs; and the occasion rendered possible a detailed study of the rates of rise and fall of $^{131}$I in the thyroid glands of grazing animals in relation to the degree of contamination of the pastures.

The value of the $^{131}$I in the thyroid glands as a measure of the hazards imposed by contamination of terrain by troposphere fall-out supervening on atomic weapon tests is considered in light of the findings.

The speed with which grazing dairy cows gather radioactive debris that has been deposited on their pastures, as indicated by the rise and fall of the $^{131}$I in their thyroid glands, is discussed in relation to the hazard entailed in the passage of bone-seeking radioactive constituents of fall-out via milk to human populations.

## I. Introduction

The primary purpose of this investigation was to assess the usefulness of the $^{131}$I concentration in the thyroids of grazing animals as an integrating measure of the degree of hazard entailed in the contamination of terrain by residua from atomic explosions. When it was undertaken, knowledge of the chemical state in which iodine isotopes exist in the air-borne debris was uncertain, and interpretation of the chain of events responsible for the accumulation of radioactivity in the thyroid tissue of the higher animals was confused. Most, if not all, of this iodine was thought to persist for a considerable period either as a gas or as an aerosol, and thus to find its way to the thyroids via the lungs. Were this so, the radioactivity of the iodine components of atomic fission residua from nuclear weapon tests might itself become

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a serious health hazard, as the amounts likely to be delivered to human foetal thyroids could be dangerous. Alternatively, were the radioactive iodine found in the thyroids of grazing animals derived from solid particles deposited on the pastures and absorbed via the alimentary canal, the amount accumulated in the thyroid gland would provide a useful index of the capacity of grazing animals to assimilate and to concentrate within animal products the longer-lived radioactive constituents of fall-out. The ability of grazing dairy cows, for instance, to gather the fall-out deposited over relatively large areas and secrete into their milk some of its components in a concentrated and easily assimilable form, is perhaps the most important single incident in a number of events that concentrate $^{90}$Sr and other bone-seeking radioactive constituents, and so enhance the risks associated with these particular isotopes.

Some aspects of the danger of $^{90}$Sr accumulation within the human skeleton have been considered in the report of the British Medical Research Council published as a White Paper on Hazards (1956). This document aimed to assess the possible untoward effects that fall-out from U.S.A. and U.S.S.R. megaton nuclear weapon tests may have on the inhabitants of the British Isles, and so risks imposed by radioactive material settling from the stratosphere were the primary consideration.

The degree of contamination of any part of the earth's surface by deposition of widely dispersed radioactive material from the stratosphere, however, is quite small when compared with the degree of contamination of certain areas in Australia with radioactive material deposited from the troposphere subsequent to the recent weapon tests.

The results of a survey of the amounts of $^{131}$I found in the thyroid glands of animals grazing in various parts of Australia (Fig. 1) that are reported in this paper indicate that extensive areas of Australia have been contaminated, and that some of the more heavy precipitations occurred on terrain situated over 1500 miles from the site of the explosions, in areas more or less thickly populated.

## Published Reports of Radioactivity in Thyroid Glands

In September 1954, Van Middlesworth (1954) reported that thyroid glands taken from cattle grown in various parts of U.S.A. and slaughtered at Memphis, at San Francisco, and at Boston were radioactive and that the radioactivity had the same decay and absorption characteristics as $^{131}$I. The highest concentration observed was $4 \cdot 4$ m$\mu$c $^{131}$I/g in a gland from one of a group of range-fed cattle raised in Florida; and the average of glands from animals slaughtered in Tennessee on June 16, 1954, was $0 \cdot 29$ m$\mu$c $^{131}$I/g.

Shortly afterwards, Gunther and Jones (1954) reported from the Radiation Laboratory at Berkeley measurements which confirmed the observations of Van Middlesworth. The concentrations of $^{131}$I in the thyroids measured there, extrapolated to June 16 on the assumption of an 8-day half-life, ranged between $0 \cdot 13$ and $1 \cdot 4$ m$\mu$c $^{131}$I/g with a mean of $0 \cdot 56 \pm 0 \cdot 288$ m$\mu$c $^{131}$I/g, and it was concluded tentatively that $^{131}$I, originating from nuclear weapon tests and air-borne in the interim as aerosols, had been absorbed by these animals via their lungs.

Iodine, in gaseous form or in aerosol suspensions, is known to be absorbed through the lungs with extraordinary efficiency, and the possibility that ^{131}I found its way into the body via this route was strengthened by the Californian observations which indicated a similar concentration of ^{131}I in all thyroids that were tested, irrespective of whether they were from carnivores or from grazing herbivores.



Fig. 1.—Location of areas (cf. Table 1) from which thyroid glands were collected. The circles centred on Monte Bello are c. 600 miles apart and those centred on Maralinga are c. 300 miles apart.

Later, Van Middlesworth (1956) published results of a further series of observations which focused medical attention on the danger of fall-out. Concentrations of ^{131}I estimated in glands collected at abattoirs within a radius of 200 miles from Memphis at relatively short intervals during the period extending from October 1954 to March 1956 indicated a series of peaks of concentrations of ^{131}I within the thyroids of cattle which ranged from 0·01 to 10 mμc ^{131}I/g thyroid, and which could be correlated with the American and Russian atomic explosions.

Human thyroids collected at autopsies conducted in the Memphis hospitals during this period had in them very much lower concentrations of [131]I—never more

than $0 \cdot 5$ per cent. of the maximum observed in cattle*—and so doubt was shed on the suggestion that all, or even a considerable part of the $^{131}$I that accumulates in the thyroids of grazing animals is absorbed through the lungs.

## II. Radioactive Iodine in Thyroid Glands of Sheep and Cattle Grazing in Various Parts of Australia Subsequent to the 1956 Atomic Weapon Tests

In 1956 arrangements were made for a periodic collection of thyroid glands from sheep and cattle grazing at a number of sites distributed over northern and north-eastern Australia in areas likely to be traversed by the plumes from the Maralinga series of tests. These arrangements had, of necessity, to be made by correspondence, and as security restrictions that were imposed forbade frank disclosure of the purpose of the collections, the extent of the survey was somewhat limited. However, besides settling unequivocally some questions of a more basic scientific nature, the data demonstrated the usefulness of the $^{131}$I content of the thyroid glands of grazing stock as a measure of the degree of hazard entailed in the contamination of particular areas by radioactive debris from nuclear weapons.

Collection-site locations are indicated in Figure 1. Analytical findings are summarized in Table 1. The more complete data from Adelaide and from the Alice Springs series are the basis of Figures 2, 3, 4, and 5.

Prior to mid-May all glands examined contained $<5\ \mu\mu$c $^{131}$I/g, which was the low limit of the sensitivity of the apparatus at our disposal.

A few days subsequent to the first Monte Bello explosion (May 16, 1956), activity due to $^{131}$I could be detected in thyroid glands collected from sheep and cattle depastured on areas distributed between latitudes 24° and 30°S. within a band approximately 500 miles wide stretching from the western to the eastern seaboards.

After the second Monte Bello explosion (June 19, 1956) the concentrations of $^{131}$I within the thyroids of animals in these regions increased to levels a hundredfold or more above those observed after the first explosion.† In some instances the observed concentrations at this period exceeded by approximately an order of magnitude the highest range of the $^{131}$I concentrations—the Memphis figures—reported in the literature by American observers (Van Middlesworth 1956).

The concentrations of $^{131}$I observed in the thyroids of grazing stock after the second Monte Bello explosion indicated that some of the areas most heavily contaminated with fall-out from the plume of this event were far distant—1500–2000

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* The "standard" measure of the tidal air which in the normal course of respiration is passed through the lungs of a 70-kg man (c. 20 cu. m./day) is about one-fifth of the tidal air of a grown bullock and, as the weights of the respective thyroid glands are approximately equal, the expected concentration of $^{131}$I in a human thyroid tissue would be approximately one-fifth of that found in the thyroids of cattle breathing the same air, if the $^{131}$I were absorbed through the lungs and concentrated within the respective thyroids with equal efficiency.

† At Rockhampton, for instance, thyroids collected from cattle on May 29, 1956, and June 22, 1956, contained respectively $3 \cdot 2$ and $2 \cdot 5$ m$\mu$c $^{131}$I, and on July 12, 1956, 440 m$\mu$c $^{131}$I. The explosion responsible for the low level occurred on May 16, 1956, and for the high level on June 19, 1956.

### TABLE 1

#### RADIOACTIVE IODINE CONTENT OF THYROID GLANDS OF SHEEP AND CATTLE

The procedure employed for the estimation of iodine concentration was essentially as follows: the gland was fused with KOH at 550°C, the aqueous solution of the melt was acidified with $H_2SO_4$ in presence of excess $NaNO_3$, and exhaustively extracted with $CHCl_3$, in which solvent, after washing with $NaHCO_3$, the iodine was titrated with $0\cdot01N$ $Na_2S_2O_3$ in the presence of $NaHCO_3$. Observed recoveries were over 95 per cent. A dash indicates that the sample was not analysed. A 1-in. diameter well-type scintillation counter was employed to estimate the 131I content of the thyroid tissue (usually of the whole gland). The $\gamma$-radiation was measured in a narrow (1 V) channel at the $0\cdot364$ MeV peak of the $\gamma$-energy spectrum of 131I. Prior to each set of determinations and at intervals throughout, the instrument was adjusted to the $0\cdot364$ MeV peak and calibrated against standards provided by the X-ray and Radium Laboratory, Commonwealth Department of Health. The 131I, determined in this way, was proven by $\gamma$-energy spectra and decay curves of samples periodically selected at random. There was no evidence of any radioactive substance other than 131I in the glands examined. Most measurements were made at least 14 days subsequent to the origin of the radioactive fission products of which the 131I was an integral part. The amount of 131I found on analysis was adjusted to the day of slaughter by extrapolation according to $\tau^{\frac{1}{2}} = 8$ days. In those instances where only a part of the thyroid gland was received, the figures for total 131I are low. A dash indicates that the whole gland contained $<5$ $\mu\mu c$ 131I. The two Monte Bello tests took place on 16.v.56 and 19.vi.56, and the four Maralinga tests on 27.ix.56, 4.x.56, 11.x.56, and 22.x.56 respectively

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | mμc 131I Total | mμc 131I per Gram Tissue | Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | mμc 131I Total | mμc 131I per Gram Tissue |
|---|---|---|---|---|---|---|---|---|---|
| *Site 4: Amaroo Station, N.T.* | | | | | *Site 6: Alice Springs, N.T. (Continued)* | | | | |
| Cattle | 9. v.56 | 5·2 | — | — | Cattle | 16. vii.56 | — | 39·6 | 2·2 |
| ,, | 25. vii.56 | 5·2 | 312·0 | 21·0 | ,, | 16. vii.56 | 3·0 | 42·0 | 1·4 |
| ,, | 3.viii.56 | 3·5 | 78·0 | 7·8 | ,, | 17. vii.56 | 4·0 | 31·2 | 2·2 |
| ,, | 3. ix.56 | 4·1 | 8·0 | 0·44 | ,, | 19. vii.56 | 3·6 | 19·2 | 1·1 |
| ,, | 17. x.56 | 6·3 | 35·5 | 2·2 | ,, | 19. vii.56 | — | 32·2 | 1·6 |
| ,, | 20. xi.56 | 4·6 | 104·0 | 8·4 | ,, | 23. vii.56 | 4·5 | 32·0 | 1·3 |
| *Site 5: Finke, N.T.* | | | | | ,, | 23. vii.56 | — | 19·6 | 1·1 |
| Cattle | 20. v.56 | 8·3 | — | — | ,, | 26. vii.56 | 2·9 | 32·6 | 1·63 |
| ,, | 2. vii.56 | 3·6 | 67·2 | 2·24 | ,, | 30. vii.56 | 4·9 | 19·8 | 1·6 |
| ,, | 15.viii.56 | 2·0 | 6·68 | 0·26 | ,, | 30. vii.56 | — | 24·4 | 1·0 |
| ,, | 22. ix.56 | 5·7 | 0·8 | 0·03 | ,, | 2.viii.56 | 3·3 | 15·4 | 0·7 |
| ,, | 21. x.56 | 5·1 | 19·8 | 1·1 | ,, | 2.viii.56 | — | 15·0 | 0·75 |
| ,, | 23. x.56 | 4·6 | 17·8 | 1·3 | ,, | 9.viii.56 | 4·1 | 68·0 | 2·7 |
| ,, | 21. xi.56 | 6·2 | 5·0 | 0·46 | ,, | 9.viii.56 | 3·3 | 17·5 | 1·3 |
| *Site 6: Alice Springs, N.T.* | | | | | ,, | 13.viii.56 | 3·9 | 14·5 | 0·5 |
| Cattle | 28. vi.56 | 3·4 | 33·0 | 1·65 | ,, | 16.viii.56 | 3·3 | 12·2 | 0·5 |
| ,, | 28. vi.56 | 5·8 | 67·2 | 2·24 | ,, | 20.viii.56 | 3·9 | 3·8 | 0·2 |
| ,, | 2. vii.56 | 5·6 | 38·0 | 1·9 | ,, | 23.viii.56 | 3·4 | 6·2 | 0·4 |
| ,, | 5. vii.56 | 2·2 | 28·8 | 2·0 | ,, | 27.viii.56 | 4·7 | 4·8 | 0·16 |
| ,, | 5. vii.56 | 3·1 | 38·4 | 2·6 | ,, | 30.viii.56 | 5·9 | 5·0 | 0·25 |
| ,, | 9. vii.56 | 8·6 | 39·0 | 2·2 | ,, | 30.viii.56 | 4·1 | 2·6 | 0·1 |
| ,, | 12. vii.56 | 2·8 | 40·0 | 2·5 | ,, | 3. ix.56 | 3·4 | 2·4 | 0·1 |
| | | | | | ,, | 10. ix.56 | 2·6 | 2·4 | 0·08 |
| | | | | | ,, | 13. ix.56 | 8·8 | 1·68 | 0·04 |
| | | | | | ,, | 17. ix.56 | 3·4 | 0·9 | 0·03 |
| | | | | | ,, | 24. ix.56 | 4·0 | 0·4 | 0·02 |

TABLE 1 (Continued)

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | Radioactivity of Thyroid at Time of Slaughter mμc $^{131}$I Total | mμc $^{131}$I per Gram Tissue |
|---|---|---|---|---|
| *Site 6: Alice Springs, N.T. (Continued)* | | | | |
| Cattle | 27. ix.56 | 5·3 | 0·44 | 0·02 |
| ,, | 1. x.56 | 5·9 | 0·5 | 0·035 |
| ,, | 4. x.56 | 3·8 | 1·17 | 0·05 |
| ,, | 8. x.56 | 3·9 | 2·0 | 0·09 |
| ,, | 11. x.56 | 5·4 | 10·5 | 0·34 |
| ,, | 15. x.56 | 4·3 | 10·0 | 0·42 |
| ,, | 18. x.56 | 5·0 | 6·5 | 0·44 |
| ,, | 22. x.56 | 7·3 | 8·7 | 0·41 |
| ,, | 25. x.56 | 3·6 | 8·9 | 0·41 |
| ,, | 29. x.56 | 5·2 | 28·3 | 1·0 |
| ,, | 1. xi.56 | 4·6 | 40·0 | 1·6 |
| ,, | 5. xi.56 | 5·5 | 51·0 | 1·7 |
| ,, | 8. xi.56 | 4·8 | 51·0 | 1·6 |
| ,, | 12. xi.56 | 2·8 | 29·0 | 1·2 |
| ,, | 15. xi.56 | 3·1 | 41·0 | 1·45 |
| ,, | 19. xi.56 | 2·6 | 24·0 | 0·95 |
| ,, | 22. xi.56 | 4·8 | 10·9 | 0·57 |
| ,, | 22. xi.56 | 5·3 | 16·3 | 0·81 |
| ,, | 3. xii.56 | 6·2 | 8·4 | 0·3 |
| ,, | 6. xii.56 | 4·8 | 7·0 | 0·29 |
| *Site 7: Elliott, N.T.* | | | | |
| Cattle | 7. v.56 | 3·8 | — | — |
| ,, | 25. vi.56 | 3·5 | 91·6 | 4·58 |
| ,, | 6.viii.56 | 4·4 | 17·8 | 0·9 |
| *Site 8: Quorn, S.A.* | | | | |
| Sheep | 8. v.56 | 7·5 | — | — |
| ,, | 2. vii.56 | 6·1 | 0·44 | 0·11 |
| ,, | 2.viii.56 | 2·9 | 1·5 | 0·5 |
| ,, | 30.viii.56 | 2·2 | 0·5 | 0·07 |
| ,, | 27. ix.56 | 4·6 | 0·18 | 0·04 |
| ,, | 23. x.56 | 2·1 | 6·6 | 2·0 |
| *Site 9: Cleve, S.A.* | | | | |
| Sheep | 29. iv.56 | 6·6 | — | |
| ,, | 29. vi.56 | 7·8 | — | — |
| Cattle | 1.viii.56 | 3·3 | 3·4 | 0·18 |
| Sheep | 3. ix.56 | 4·4 | — | — |
| ,, | 11. x.56 | 8·2 | 1·0 | 0·18 |
| ,, | 31. x.56 | 7·7 | 14·0 | 5·8 |

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | Radioactivity of Thyroid at Time of Slaughter mμc $^{131}$I Total | mμc $^{131}$I per Gram Tissue |
|---|---|---|---|---|
| *Site 10: Animals Slaughtered at Brisbane Abattoirs* | | | | |
| Cattle | 28. v.56 | 6·0 | 2·8 | 0·1 |
| ,, | 3. vii.56 | 6·4 | 22·5 | 1·2 |
| ,, | 26. vii.56 | 4·0 | 31·8 | 1·9 |
| ,, | 9.viii.56 | 2·6 | 9·2 | 0·4 |
| ,, | 2. x.56 | 5·0 | 1·18 | 0·07 |
| ,, | 29. x.56 | 4·8 | 7·2 | 0·36 |
| ,, | 12. xi.56 | 6·4 | 16·2 | 0·65 |
| *Site 11: Blackall, Qld.* | | | | |
| Sheep | 8. v.56 | 2·0 | — | — |
| ,, | 27. vii.56 | 5·2 | 30·0 | 6·6 |
| ,, | 19.viii.56 | 2·7 | 3·6 | 1·2 |
| ,, | 7. ix.56 | 5·4 | 0·8 | 0·2 |
| ,, | 31. x.56 | 3·6 | 86·0 | 31·0 |
| *Site 12: Charleville, Qld.* | | | | |
| Sheep | 15. v.56 | 3·7 | — | — |
| ,, | 29. vii.56 | 5·5 | 38·0 | 12·5 |
| ,, | 23.viii.56 | 2·8 | 5·0 | 1·2 |
| ,, | 18. ix.56 | 4·0 | — | — |
| ,, | 22. x.56 | 3·5 | 14·4 | 4·8 |
| ,, | 16. xi.56 | 1·8 | 23·0 | 4·6 |
| *Site 13: Cunnamulla, Qld.* | | | | |
| Cattle | 22.viii.56 | 5·1 | 1·6 | 0·1 |
| *Site 14: Julia Creek, Qld.* | | | | |
| Sheep | 16. v.56 | 4·6 | — | — |
| ,, | 13. vii.56 | 6·0 | 144·0 | 70·0 |
| ,, | 15.viii.56 | 2·6 | 7·1 | 1·4 |
| ,, | 26. ix.56 | 6·3 | 0·41 | 0·09 |
| ,, | 1. x.56 | 3·0 | 2·57 | 0·51 |
| ,, | 23. xi.56 | 3·9 | 74·0 | 13·4 |
| *Site 15: Longreach, Qld.* | | | | |
| Sheep | 3. v.56 | 3·0 | — | — |
| ,, | 4. vii.56 | 1·4 | 131·0 | 44·0 |
| ,, | 25. vii.56 | 3·0 | 43·0 | 8·6 |

TABLE 1 (*Continued*)

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | mμc $^{131}$I Total | mμc $^{131}$I per Gram Tissue |
|---|---|---|---|---|
| *Site 15: Longreach, Qld.* (Continued) | | | | |
| Sheep | 7. ix.56 | 2·2 | 1·0 | 0·25 |
| ,, | 28. ix.56 | 1·7 | — | — |
| ,, | 9. xi.56 | 1·0 | 101·0 | 26·0 |
| *Site 16: Mt. Isa, Qld.* | | | | |
| Cattle | 28. ix.56 | 4·5 | 5·8 | 0·38 |
| ,, | 7. xi.56 | 3·9 | 181·0 | 7·5 |
| *Site 17: Rockhampton, Qld.* | | | | |
| Cattle | 29. v.56 | 4·8 | 3·2 | 0·15 |
| ,, | 22. vi.56 | 6·2 | 2·5 | 0·15 |
| ,, | 12. vii.56 | 2·0 | 440·0 | 17·5 |
| ,, | 9.viii.56 | 5·3 | 18·7 | 0·89 |
| ,, | 20. ix.56 | 5·9 | 0·6 | 0·03 |
| ,, | 30. x.56 | 4·8 | 47·0 | 2·2 |
| ,, | 9. xi.56 | 4·6 | 57·0 | 3·8 |
| *Site 18: Townsville, Qld.* | | | | |
| Cattle | 29. vii.56 | 2·3 | 29·4 | 1·47 |
| ,, | 17.viii.56 | 4·2 | 5·2 | 0·26 |
| ,, | 18. ix.56 | 4·5 | 0·88 | 0·03 |
| ,, | 16. x.56 | 7·2 | 22·0 | 0·86 |
| ,, | 30. xi.56 | 6·7 | 88·0 | 5·4 |
| *Site 19: Winton, Qld.* | | | | |
| Sheep | 13. v.56 | 1·8 | — | — |
| ,, | 8.viii.56 | 3·7 | 7·5 | 5·0 |
| ,, | 9.viii.56 | 5·1 | 2·9 | 0·7 |
| ,, | 10. ix.56 | 1·3 | — | — |
| *Site 20: Animals Slaughtered at Sydney Abattoirs* | | | | |
| Cattle | 26. vi.56 | 5·5 | — | — |
| ,, | 23. vii.56 | 7·0 | 0·9 | 0·05 |
| ,, | 26. ix.56 | 6·4 | 0·2 | 0·005 |
| *Site 21: Broken Hill, N.S.W.* | | | | |
| Sheep | 20. v.56 | 1·8 | 0·42 | 0·08 |
| ,, | 3. vii.56 | 4·2 | — | — |

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | mμc $^{131}$I Total | mμc $^{131}$I per Gram Tissue |
|---|---|---|---|---|
| *Site 22: Bourke, N.S.W.* | | | | |
| Sheep | 31. v.56 | 2·5 | 0·53 | 0·1 |
| ,, | 17. vi.56 | 3·7 | 0·23 | 0·125 |
| ,, | 17. vii.56 | 3·7 | 6·0 | 1·5 |
| ,, | 29.viii.56 | 4·9 | 0·27 | 0·09 |
| ,, | 12. xi.56 | 5·2 | 14·7 | 3·1 |
| *Site 23: Condobolin, N.S.W.* | | | | |
| Sheep | 10. vii.56 | 4·8 | 1·5 | 0·75 |
| ,, | 24. ix.56 | 3·1 | — | — |
| ,, | 23. x.56 | 5·0 | 5·3 | 1·4 |
| ,, | 30. xi.56 | 8·2 | 0·27 | 0·12 |
| *Site 24: Narrandera, N.S.W.* | | | | |
| Sheep | 31. vii.56 | 5·3 | 0·8 | 0·3 |
| ,, | 30.viii.56 | 4·2 | 0·4 | 0·06 |
| ,, | 3. x.56 | 3·7 | — | — |
| ,, | 29. x.56 | 5·4 | 3·8 | 1·2 |
| ,, | 4. xii.56 | 6·3 | 0·17 | 0·03 |
| *Site 25: Hay, N.S.W.* | | | | |
| Cattle | 16. vii.56 | 3·2 | 0·92 | 0·04 |
| Sheep | 16. vii.56 | — | 1·01 | 0·17 |
| Cattle | 21. ix.56 | 5·1 | — | — |
| Sheep | 21. ix.56 | 6·6 | — | — |
| Cattle | 23. x.56 | 5·6 | 22·0 | 0·55 |
| Sheep | 23. x.56 | — | 17·5 | 3·8 |
| Cattle | 27. xi.56 | 5·0 | 3·4 | 0·2 |
| Sheep | 27. xi.56 | 4·3 | 0·50 | 0·12 |
| *Site 26: Monkira Station, Qld.* | | | | |
| Cattle | 21. vii.56 | — | 20·7 | 1·22 |
| ,, | 19.viii.56 | — | 4·04 | 0·16 |
| ,, | 29. ix.56 | 3·3 | 4·5 | 0·26 |
| ,, | 9. x.56 | 3·3 | 830·0 | 37·0 |
| ,, | 12. xi.56 | 4·7 | 353·0 | 16·1 |

TABLE 1 (Continued)

### Site a: Animals Slaughtered at Adelaide Abattoirs

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | mμc $^{131}$I Total | mμc $^{131}$I per Gram Tissue |
|---|---|---|---|---|
| Cattle | 15. v.56 | 5·1 | — | — |
| ,, | 30. v.56 | 4·8 | 2·3 | 0·115 |
| ,, | 18. vi.56 | 7·1 | 9·6 | 0·34 |
| ,, | 18. vi.56 | — | 2·2 | 0·04 |
| Sheep | 26. vi.56 | 4·2 | — | — |
| Cattle | 6. vii.56 | 5·1 | 16·6 | 0·64 |
| ,, | 9. vii.56 | — | 20·8 | 1·7 |
| Sheep | 11. vii.56 | — | 0·8 | 0·26 |
| Cattle | 11. vii.56 | 6·1 | 3·75 | 0·23 |
| Sheep | 11. vii.56 | — | 0·49 | 0·16 |
| ,, | 11. vii.56 | — | 0·63 | 0·21 |
| ,, | 11. vii.56 | — | 0·60 | 0·4 |
| Cattle | 11. vii.56 | — | 9·1 | 0·35 |
| Sheep | 18. vii.56 | — | 0·79 | 0·13 |
| Cattle | 2.viii.56 | 4·9 | 10·4 | 0·3 |
| ,, | 2.viii.56 | — | 6·2 | 0·3 |
| Sheep | 2.viii.56 | — | 2·9 | 0·4 |
| ,, | 2.viii.56 | — | 2·4 | 0·5 |
| ,, | 8.viii.56 | — | 1·15 | 0·6 |
| ,, | 8.viii.56 | — | 0·8 | 0·4 |
| Cattle | 13.viii.56 | 4·7 | 1·07 | 0·04 |
| ,, | 11. ix.56 | 4·4 | — | — |
| ,, | 27. ix.56 | 6·3 | 0·46 | 0·02 |
| ,, | 23. xi.56 | 4·3 | 11·8 | 0·76 |
| ,, | 23. xi.56 | 7·0 | 11·9 | 0·63 |
| ,, | 28. xi.56 | 5·4 | 4·0 | 0·20 |
| ,, | 28. xi.56 | 7·0 | 5·4 | 0·27 |

### Glenthorne, Adelaide

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | mμc $^{131}$I Total | mμc $^{131}$I per Gram Tissue |
|---|---|---|---|---|
| Sheep | 26. vi.56 | — | — | — |
| ,, | 28. vi.56 | — | — | — |
| ,, | 5. vii.56 | — | — | — |
| ,, | 12. vii.56 | — | — | — |
| ,, | 17. x.56 | — | 43·0 | 8·4 |
| ,, | 18. x.56 | — | 33·0 | 11·0 |
| ,, | 19. x.56 | — | 32·0 | 9·6 |
| ,, | 20. x.56 | — | 36·0 | 11·0 |
| ,, | 22. x.56 | — | 20·0 | 8·3 |
| ,, | 24. x.56 | 5·7 | 29·5 | 8·4 |
| ,, | 26. x.56 | — | 23·0 | 8·3 |

### Glenthorne, Adelaide (Continued)

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | mμc $^{131}$I Total | mμc $^{131}$I per Gram Tissue |
|---|---|---|---|---|
| Sheep | 29. x.56 | — | 15·5 | 5·4 |
| ,, | 31. x.56 | 4·4 | 24·0 | 5·5 |
| ,, | 2. xi.56 | — | 15·0 | 4·9 |
| ,, | 2. xi.56 | — | 17·5 | 5·7 |
| ,, | 5. xi.56 | — | 12·3 | 3·2 |
| ,, | 8. xi.56 | — | 2·3 | 1·1 |
| ,, | 12. xi.56 | — | 4·1 | 1·3 |
| ,, | 15. xi.56 | — | 2·5 | 0·88 |
| ,, | 19. xi.56 | — | 4·2 | 0·93 |
| ,, | 22. xi.56 | — | 2·4 | 0·48 |
| ,, | 26. xi.56 | — | 1·15 | 0·43 |
| ,, | 29. xi.56 | — | 0·4 | 0·14 |
| ,, | 3. xii.56 | — | 0·91 | 0·15 |
| ,, | 10. xii.56 | — | 0·34 | 0·11 |

### Roseworthy, Adelaide

| Animal | Date of Slaughter | I in Thyroid (mg I/g dry wt.) | mμc $^{131}$I Total | mμc $^{131}$I per Gram Tissue |
|---|---|---|---|---|
| Sheep | 30. x.56 | — | 19·8 | 6·6 |
| ,, | 30. x.56 | — | 12·0 | 5·5 |
| ,, | 30. x.56 | — | 19·4 | 8·0 |
| ,, | 30. x.56 | — | 11·0 | 5·1 |
| ,, | 30. x.56 | — | 18·0 | 3·9 |
| ,, | 5. xi.56 | — | 6·3 | 3·0 |
| ,, | 13. xi.56 | — | 5·3 | 1·8 |
| ,, | 13. xi.56 | — | 4·7 | 1·5 |
| ,, | 13. xi.56 | — | 4·0 | 1·1 |
| ,, | 13. xi.56 | — | 6·6 | 1·9 |
| ,, | 13. xi.56 | — | 5·9 | 1·3 |
| ,, | 13. xi.56 | — | 4·4 | 1·3 |
| ,, | 13. xi.56 | — | 2·8 | 0·9 |
| ,, | 13. xi.56 | — | 5·9 | 1·9 |
| ,, | 19. xi.56 | — | 3·2 | 0·73 |
| ,, | 19. xi.56 | — | 4·8 | 1·50 |
| ,, | 19. xi.56 | — | 2·2 | 0·85 |
| ,, | 19. xi.56 | — | 2·15 | 0·90 |
| ,, | 19. xi.56 | — | 2·6 | 0·96 |
| ,, | 27. xi.56 | 9·3 | 1·52 | 0·53 |
| ,, | 27. xi.56 | — | 0·92 | 0·40 |
| ,, | 6. xii.56 | — | 0·6 | 0·14 |
| ,, | 6. xii.56 | — | 0·6 | 0·12 |
| ,, | 6. xii.56 | — | 0·42 | 0·085 |
| ,, | 6. xii.56 | — | 0·44 | 0·12 |

miles away—from the weapon test site, cf. Table 1.  The subsequent effects of this explosion could be detected in the thyroids of grazing animals depastured within a band of terrain about a thousand miles wide, stretching west to east across the Australian continent.  The variability observed within this band emphasized the uneven distribution of the deposited radioactive debris.
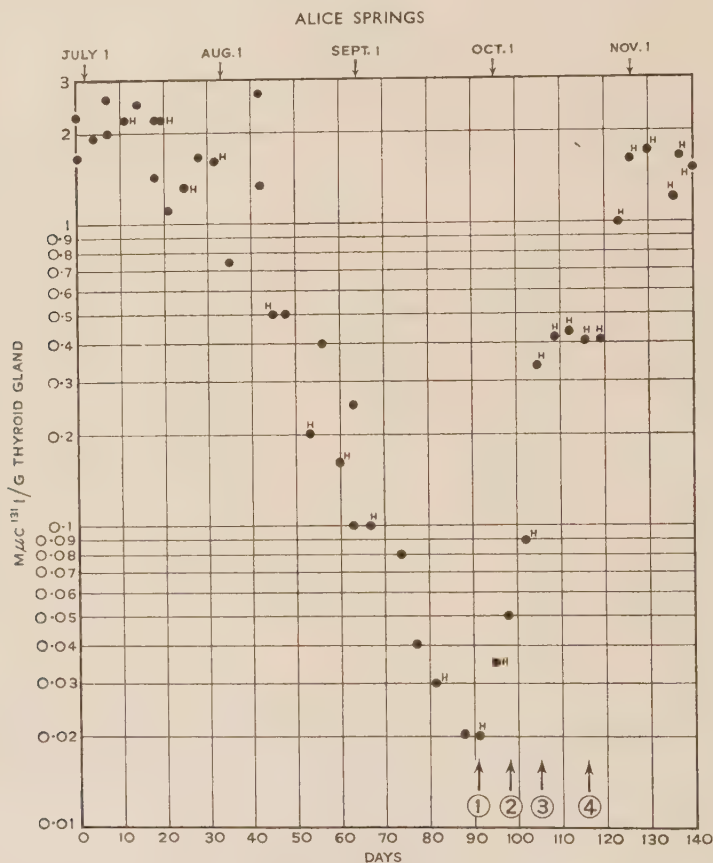


Fig. 2.—The ^{131}I concentrations of thyroid glands collected from cattle grazed in the vicinity of Alice Springs are set out in relation to the dates of the atomic explosions from which the debris containing the radioactive iodine was derived.  The activity at the beginning of the observations reported here was the result of contamination originating on June 19, 1956, at Monte Bello.  The accumulation in the thyroids of cattle grazed in the region of Alice Springs was about one-tenth of that observed at this period c. 1200 miles east at the same latitude, cf. Table 1.  The dates of the four subsequent tests at Maralinga are indicated by serial numbers. Data marked H are from cattle grazed at Hamilton Downs Station.

Areas on the north-eastern seaboard and in central western Queensland that received the relatively heavy dressings from the Monte Bello tests are within the sector over which prevailing winds tended to carry the plumes from tests conducted later at Maralinga.  In these latter areas the observations, Table 1, indicate repeated dressings from subsequent weapon tests.

## The Alice Springs Series

Throughout the period extending from June 28 to mid-December 1956, thyroid glands were taken at intervals of c. 3 days from cattle that had grazed within the vicinity of Alice Springs. The data from this frequent sampling, set out in Figure 2, allow certain generalizations to be drawn.

Although the concentrations of $^{131}$I observed in the thyroids of cattle at this site were never particularly high if compared with those found in thyroids taken from cattle grazed during this period in the same latitudes 700–1000 miles east, a noteworthy feature of the series is that after the second Monte Bello explosion, the concentrations of $^{131}$I in the thyroid glands remained, within the expected limits of variance, relatively constant for a period of about 40 days before beginning to decrease exponentially.*

The accumulation and persistence of $^{131}$I within the thyroids of grazing animals are influenced by a number of variables. In this instance, losses of $^{131}$I due to metabolic turnover of iodine in the thyroid and to the radioactive decay of the $^{131}$I deposited in the gland were made good over a period of about five half-lives of $^{131}$I by additional $^{131}$I assimilated from the fodder, which was itself a decaying source. The concentration of $^{131}$I on the fodder plants would be determined during this interval by radioactive decay of $^{131}$I to stable $^{131}$Xe and, to some degree in the very early stages, by renewal from the precursor $^{131}$Te. Neither wind and rain, which would remove, in part, adherent particles of radioactive debris from the fodder plants, nor the growth of the fodder plants themselves, which would, in effect, dilute the radioactive contamination of the material taken by the grazing animals, exerted any very considerable influence there during the 40 days subsequent to this precipitation. This is in distinct contrast to what happened in other circumstances in the vicinity of Adelaide (cf. Fig. 5).

The effects of contamination of the Alice Springs area by debris from the first, second, and fourth Maralinga explosions, clearly discernible in the data, express the fact that the surrounding country had been subject to at least four dressings of radioactive material from atomic explosions. The hazards of successive contaminations are cumulative.

## The Adelaide Fall-out

We, in this laboratory, have been interested for some time in the radon ($^{222}$Rn) and thoron ($^{220}$Rn) content of soil atmospheres, and in the radioactivity of the air which in greater part is due to daughter products† of these emanations from the rocks and soils (Stout, Jones, and Delwiche 1957). Periodic series of estimations of radioactivity of Adelaide air that were made were not a continuous routine, but on no occasion before October 13, 1956, did chemical separations and measurements of decay characteristics of the radioactive material collected in the course of these

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* The kinetics responsible for this apparent pause in the decay of the $^{131}$I in the thyroids is discussed in the legend to Figure 5.

† The radioactivity of the air in Adelaide is made up mainly of $^{212}$Pb, $^{214}$Pb, and their degradation products, which adhere to dust particles that may be collected by drawing air through a filter paper (Stout et al. 1957).

studies suggest any considerable contamination from isotopes derived from explosions of nuclear weapons. However, the very large increase in the amount of radioactivity in the 24-hr catch (October 12–13) from the filtration of 20 cu. m. of air clearly indicated that the plume from the third Maralinga explosion (October 11) passed close to Adelaide and contaminated the city and surrounding country with radioactive fission products. The radioactivities of the particulate matter caught on the filter-paper disks during the week October 12–18 are set out in Table 2.

TABLE 2

ADELAIDE FALL-OUT FROM THE THIRD MARALINGA EXPLOSION

Radioactivity in tidal air (20 cu. m.)

| Collection Period October 1956 | Prevailing Wind | Rain (in.) | Activity on Filter Pad* (counts/100 sec) |
|---|---|---|---|
| 12–13 | N.–NW. | Nil | 95,000 |
| 13–14 | N.–NW. | Nil | 11,200 |
| 14–15 | NW. | 0·29 | 1,030 |
| 15–16 | SW. | Nil | 3,850 |
| 16–17 | SW. | 0·09 | 350 |
| 17–18 | SW. | 0·36 | 21 |
| 19–20 | SW. | Nil | 62 |

* Collected by drawing c. 20 cu. m. air through a $\frac{7}{8}$-in. disk of filter paper at a steady rate over 24 hr. Radioactivity was estimated immediately after completing the collection by placing the disk 1 mm from the end window of an E.M.H.2 tube of which the counting efficiency for $^{40}K$ standard under similar conditions was $\leqslant 15$ per cent. The tidal air is the volume of air taken into and expelled from the lungs in the normal course of breathing. A 70 kg man breathes c. 20 cubic metres a day.

The greater part of the precipitation occurred on two rainless days; on the third day, October 15, about 0·3 in. of rain fell in gusty showers. The radioactivity of this rain was not determined.

This occasion provided an opportunity to study more intensively the effects of a precipitation of radioactive debris, for the Division's field station, Glenthorne, a square mile of country situated 11 miles south of Adelaide, was in the contaminated area.

The thyroid gland removed on October 17 from a sheep that had been grazing as a member of a flock depastured there contained 22 mμc $^{131}I$. Prior to October 12, the thyroid glands of individuals of this flock contained $<5$ μμc $^{131}I$ which was the lower limit of our means of estimation. Observations started a day or so later have provided data particularly cogent to the assessment of the hazard from fall-out.

The capacity of grazing animals to concentrate radioactive isotopes from the contaminated pastures was assessed by measurements of the radioactivity in the rumen contents of sheep drawn from the flock at intervals of 2 days; the rise and

fall of radioactivity in various organs and tissues was studied in these animals over the ensuing 2 months; and, later, observations were made at less frequent intervals.
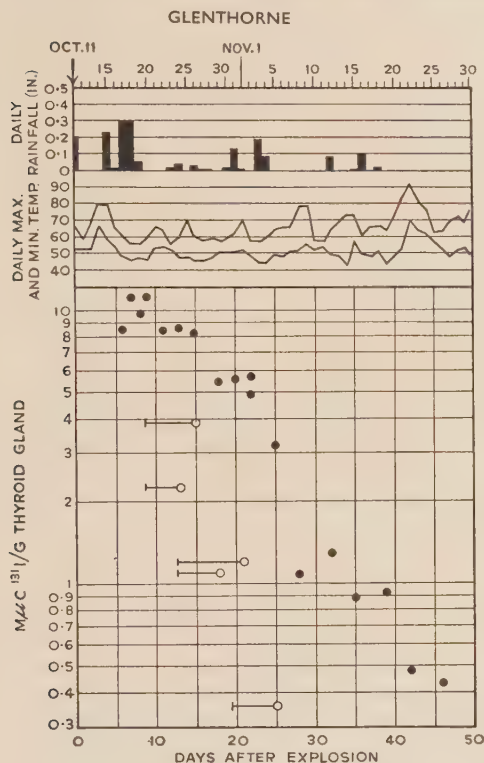


Fig. 3.
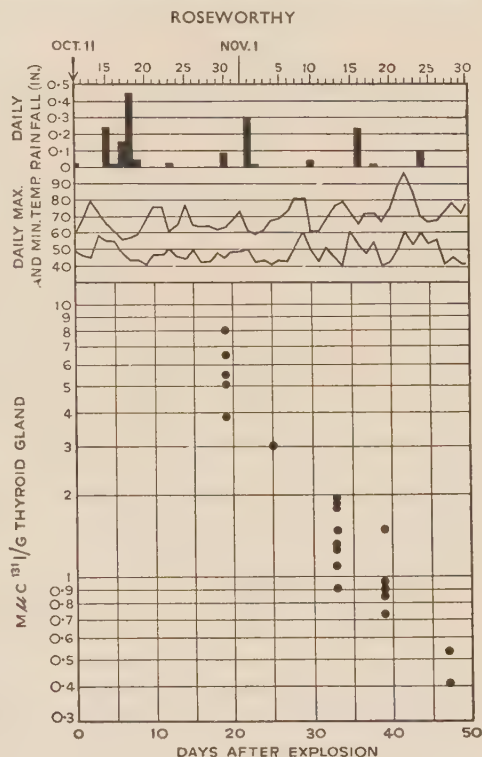
Fig. 4.

Fig. 3.—The $^{131}$I contents of the thyroid tissues of sheep of similar age grazed as a flock at Glenthorne, 11 miles south of Adelaide, are set out in relation to the time subsequent to the explosion on October 11, 1956, at Maralinga, c. 600 miles distant, from which the radioactive material that contaminated the pastures originated. The $^{131}$I concentrations represented by open circles are those found in sheep transferred from pens to the contaminated pastures on October 19 (2), on October 23 (2), and October 30 (1) and allowed to graze there with the flock until slaughtered on the days indicated.

Fig. 4.—The $^{131}$I contents of the thyroid tissues of sheep grazed as a flock at Roseworthy Agricultural College, 35 miles north of Adelaide, are set out in relation to the time subsequent to the explosion on October 11, 1956, at Maralinga (cf. Fig. 3). From analysis of variance of the findings on October 30, November 13, November 19, and December 6 (data of final date not shown in figure), the mean logs of the concentrations of $^{131}$I (m$\mu$c/g) and their standard deviations on these dates were, respectively, $0 \cdot 7523 \pm 0 \cdot 0523$, $0 \cdot 1480 \pm 0 \cdot 0442$, $-0 \cdot 0189 \pm 0 \cdot 0523$, and $-0 \cdot 9415 \pm 0 \cdot 0585$.

Examination of rumen contents taken from animals slaughtered on October 18—the first of this series—indicated that a grazing sheep collected from the Glenthorne pastures on that day sufficient radioactive material to register close to $10^4$ decompositions per second.* Radiometrical examination of this material proved

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* Estimated by measuring rumen samples with an end-window (E.M.H.2) tube. The rumen contents weighed 950 g dry wt.

its identity with that of the "catch" filtered from the atmosphere in Adelaide on October 12–13. The decay of the radioactivity in the rumen contents of animals slaughtered subsequently proceeded at a rate similar to the rate of decay of the catch. The radiometrical data leave no reasonable doubt that, throughout this series of observations at least, the material from which the $^{131}$I was derived was the same as the complex mixture of radioactive isotopes separated from the atmosphere.

Previously, we had obtained thyroid glands of cats, dogs, and rats that had lived in areas contaminated with fall-out, and had found very little or no $^{131}$I in them at a time when the concentration of $^{131}$I in the thyroids of herbivora grazing in the vicinity was high. The tentative conclusion from these observations was confirmed on October 18 *et seq.* by measurements of the radioactivity of thyroid glands from sheep drawn from a group that for some months previously had been confined in pens and fed on rations comprised essentially of chaffed cereal hay that, perchance, had been stored under conditions which would render contamination by fall-out very unlikely. These glands contained very little $^{131}$I indeed—less than 0·05 per cent. of the $^{131}$I found in glands from sheep of similar age that had been grazing on pastures in the immediate vicinity of the pens. The following protocol is typical of the findings:

*Adelaide Fall-out:* $^{131}$I *in Thyroids of Pen-fed and Pasture-fed Sheep*

*Location.*—Glenthorne, the central field station of the Division of Biochemistry and General Nutrition, C.S.I.R.O., situated 11 miles south of Adelaide.

*Previous History of Animals.*—The animals were wether hoggets selected, at random, respectively from pen-fed and pasture-fed groups. They were closely related and of similar age (*aet.* 17 months). One, A5–071, was from a group that for some months previously had been confined to a suite of roofed pens, enclosed on three sides, and freely open to the east. During this period these wethers had been fed on dry rations (comprised essentially of cereal hay produced on the property) provided in covered food troughs, and they were watered from troughs situated under the roofed part of the pens. The other, A5–391, was one of the flock from which the pen-fed group had been drawn. This flock was grazed on mixed pastures in the immediate vicinity of the pens. It was the height of spring; the pastures were particularly lush and growing rapidly.

On the morning of October 18 (i.e. 6 days after Adelaide and its environs had been contaminated with fall-out from the third Maralinga explosion) the animals were conveyed to the laboratory, slaughtered, and the $^{131}$I in their thyroids determined.

*Estimation of* $^{131}$I.—Time 11·00–12 noon, October 18, 1956, Adelaide. Instrument calibration: discriminator bias 20 V = 0·364 MeV peak of $^{131}$I $\gamma$-emission; channel width 1 V; E.H.T. 950 V; rise 0·03 $\mu$sec; fall 3 $\mu$sec; atten. 30 dB, at which setting background was 16·6 $\pm$ 1·8 counts/100 sec, and $^{131}$I std. = 272 × 10$^3$ counts/100 sec/$\mu$c $^{131}$I.

|  | Sheep No. A5–071 (pen-fed) | Sheep No. A5–391 (pasture-fed) |
|---|---|---|
| Weight of thyroid (g wet wt.) | 2·8 | 2·9 |
| Net counts/100 sec | 7 $\pm$ 3·2 | 9065 $\pm$ 18 |
| Total $^{131}$I in gland (m$\mu$c) | c. 0·025 | 33·0 |
| $^{131}$I/g thyroid (m$\mu$c) | <0·01 | 11·0 |

The decay characteristics and the energy spectrum of the $\gamma$-radiation of the gland from A5–391 were those of $^{131}$I.

Thus it is probable that only a negligible proportion of the ^{131}I observed in the thyroid glands of cattle and sheep grazed on the contaminated areas considered in this report found its way into the animals via their lungs, and so, provided the age



ALICE SPRINGS (CATTLE)          ADELAIDE (SHEEP)
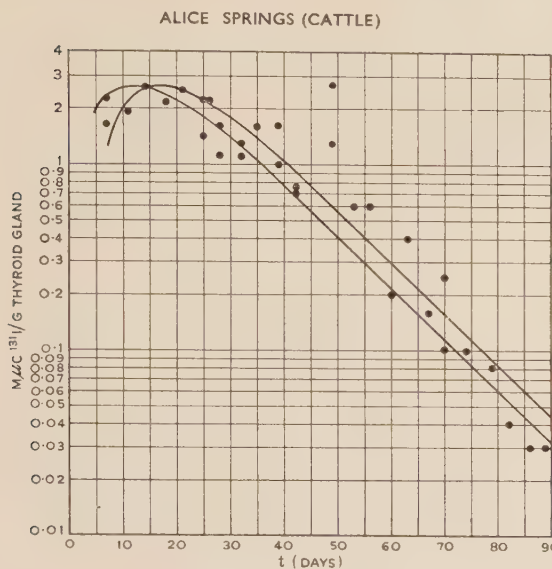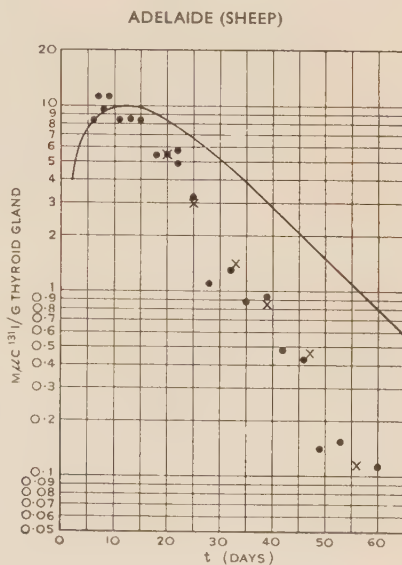
Fig. 5 (a)                      Fig. 5 (b)

Fig. 5.—The amount, $x_0$, of ^{131}I deposited at $t = 0$ on the fodder consumed in a day by a grazing animal becomes $x_0 e^{-\lambda t}$ units at time $t$, where $\lambda$ is the ^{131}I decay constant, $0 \cdot 0866$; and so, if the animal's daily intake of contaminated fodder, and the efficiency of retention of ^{131}I are constant, and the biological turnover of ^{131}I is ignored, the rate of accumulation of ^{131}I in the animal's thyroid, $dx/dt$, at time $t$ is proportional to

$$x_0 e^{-\lambda t} - \lambda x,$$

where

$$x = x_0 t e^{-\lambda t}.$$

This relationship expresses the extreme case in which the original concentration of fall-out on the fodder is not reduced by weathering, etc.

(a) Within an expected variance, the observed data of the Alice Springs series fit tolerably well the curve derived from the above relationship. To allow time for the troposphere cloud to travel from the weapon test site to the area where the observations were made, $t_0$ on the scale has been advanced 2 days from June 19, 1956. Zero of the second curve has been advanced (arbitrarily) a further 5 days. No rain fell in this area between July 16, 1956 (day 25) and September 2, 1956 (day 73).

(b) The concentrations of ^{131}I observed in the Adelaide series diminished with time much more rapidly than those implied by the curve derived from the above postulates. In this instance there were heavy falls of rain (cf. Figs. 3 and 4) and considerable growth of the pastures during the period in which the observations were made. Individual observations at Glenthorne (●) and means of observations at Roseworthy (×).

of the fall-out is known, the concentration of ^{131}I which accumulates in the thyroid gland is an expression of the rate at which the grazing animal gathers radioactive material from contaminated pastures.

The concentrations of $^{131}$I in the thyroid glands of sheep grazed at Glenthorne (11 miles south of Adelaide) and at Roseworthy College (35 miles north of Adelaide) are set out respectively in Figures 3 and 4. Further details are reported in the legends to these figures.

Conditions at Glenthorne allowed an estimate of the rate of decrease of the $^{131}$I concentrations of the pasture to be made by periodically transferring sheep from pens to the pastures and allowing them to graze there for intervals of 4–7 days before they were slaughtered. Conditions at Roseworthy, where one day each week a number of animals was slaughtered to provision the college, allowed an estimate to be made of the variance of the $^{131}$I content of the thyroids of animals grazing as a flock under identical conditions. Similarity of the two series indicated that these pastures situated about 40 miles apart had been contaminated to an almost identical degree.

In contrast to the considerable period during which the $^{131}$I concentration in glands from cattle grazed in the vicinity of Alice Springs remained nearly stationary (cf. Figs. 2, 5(a)) the concentration of $^{131}$I in the glands of sheep subsequent to the Adelaide fall-out began almost at once to decrease exponentially at a much more rapid rate than would be implied if the intake of the radioactive fall-out remained approximately constant (cf. Fig. 5(b)). The pastures in the environs of Adelaide were contaminated at the height of spring when the growth of the fodder plants was itself rapid enough to, in effect, decrease materially the overall concentration of the radioactive particles that had been deposited on them. Heavy showers of rain which fell from time to time during the early stages of the observations no doubt decreased the concentration further by washing from the pastures the adherent radioactive particles of which $^{131}$I was an integral part. Dilutions arising from these and possibly from other causes reduced the rate of intake of $^{131}$I to an extent that was insufficient to make good radioactive decay and biological losses.

The situation observed at Alice Springs after the Monte Bello exercises (Fig. 5(a)), is representative of more serious conditions than those which prevailed in the environs of Adelaide after the third Maralinga explosion (Fig. 5(b)), for a sustained $^{131}$I concentration in the thyroid under these conditions is an indication that radioactive debris remains on the pastures for a considerable period before it is in part removed to the soil from which intake is slower.

## III. Discussion

The observations set out in the body of this paper emphasize the speed with which grazing animals assimilate and concentrate $^{131}$I from constituents of the fission products that become deposited on pasture in areas traversed by the clouds of debris arising from atomic explosions. In these circumstances it may reasonably be assumed that a rapid accumulation of $^{131}$I in the thyroids of grazing cattle indicates a rapid gathering of $^{89}$Sr, $^{90}$Sr, and of other bone-seeking isotopes, and a speedy launching of these radioactive substances, via milk, into human foodstuffs, thence to the skeleton where they become deposited preferentially at the sites where mineralization is proceeding. As the intensity of radiation from the relatively

concentrated aggregates of radioactive material that are formed in this way is probably the prime determinant in the induction of the changes within the cells of the bone matrix and of the bone marrow that lead ultimately to osteosarcoma or to leukaemia, the hazard of neoplasm production would be influenced to an important degree by the rate at which these radioactive isotopes are ingested, for this determines the speed with which they are presented to the site of osteogenesis* and so the degree of their aggregation.

The hazard would be greatest during the periods in which the radioactive debris remains on the pastures, for then both the rate of ingestion and the ratios of $^{89}$Sr, $^{90}$Sr, etc. to calcium within the pastures are greatest and so the rate of transfer to human beings is at its peak.

In addition to the biological influences that tend to concentrate $^{90}$Sr within human foodstuffs, particularly within milk, there are purely physical influences that tend to increase the ratio of $^{90}$Sr to other radioactive constituents in the debris that moves off as a cloud from the site of test explosions. $^{90}$Sr comprises only about $0\cdot1$ per cent. of the original mass 90 yield of primary fission products; and its immediate precursor, $^{90}$Rb (half-life $2\cdot7$ min) only about 15 per cent. (Steinberg and Glendenin 1955; Martell 1956). The parent, $^{90}$Kr (half-life 33 sec), a noble gas, is produced in high independent yield† and is sufficiently long-lived and chemically unreactive to ensure that the greater part of its daughters, $^{90}$Rb and $^{90}$Sr, escape complex formation with vaporized silica in the fire-ball of ground level explosions: and so, after the larger particles condense from the cooling gases and shower close to the site of the explosion, the cloud of finer debris that moves off contains a considerably higher proportion of $^{90}$Sr (Martell 1956) in chemical states readily assimilable by living organisms.‡ For this reason an enrichment factor should be introduced into calculations that aim to assess the overall ingestion of $^{90}$Sr by grazing animals from the $^{131}$I found in their thyroid glands.

In the fission products at the time of the explosion, the atomic ratios§ of potential $^{90}$Sr/$^{131}$I, of potential $^{89}$Sr/$^{131}$I, and of potential $^{89}$Sr+$^{90}$Sr/$^{131}$I are $c.$ $1\cdot6$, $2\cdot0$, and $3\cdot6$ respectively, and these ratios increase with time according to

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* As the process of osteogenesis is particularly intensified in the very young, the risk is greater in foetal and neo-natal subjects.

† Reed (1955) reported a yield of $^{90}$Sr, which, based on a half-life of 28 years, corresponds to a $5\cdot6$ per cent. yield of this isotope in the fission products from $^{235}$U: thus 146 c of $^{90}$Sr are produced per $10^{12}$ kcal (1 kilo-ton T.N.T.) of energy released. The yield of $^{90}$Sr from fission of $^{238}$U and of $^{239}$Pu would be less.

‡ Some figures have been published which, indicate the extent of the error this enrichment introduces into predictions of the quantity of $^{90}$Sr based on overall radioactivity of fall-out. At Binghampton, N.Y., for instance, the amount of $^{90}$Sr separated chemically from the soil was $c.$ four times that predicted from radiometric measurement of fall-out collected on sticky paper. Similar observations on other areas indicated frequently an enrichment factor of $c.$ 2 (Eisenbud and Harley 1956).

§ Fission of $^{235}$U induced by thermal neutrons yields $4\cdot8$ per cent. of $^{89}$Sr (half-life 53 days) (Reed and Turkevich 1953), $5\cdot8$ per cent. of $^{90}$Sr (half-life 28 years) (Glendenin *et al.* 1956), and $2\cdot9$ per cent. of $^{131}$I (half-life $8\cdot1$ days) (Coryell and Sugarman 1951). Published data relating to fast neutron-induced fission is not so complete. The ratios mentioned in the text would not be altered materially in the fission products of $^{233}$U or of $^{239}$Pu.

the age of the fission products and the rates of decay of the respective isotopes. Thus for a period after pastures have been contaminated by fission products of known age, an approximation of the extent of the ingestion of $^{89}$Sr, $^{90}$Sr, and of other bone-seeking isotopes by a grazing animal might be derived directly from the $^{131}$I content of its thyroid gland, provided the efficiency of retention of ingested $^{131}$I and the rate of physiological turnover of iodine by the particular species of animal employed are known. The period of usefulness of this means of assessing the intake of the constituents of fall-out that determine the primary hazard would be limited by the rate of decay of $^{131}$I to approximately 3 months after the explosion from which the fission products originated.

## IV. ACKNOWLEDGMENTS

It is a pleasure to record my thanks to my colleagues, Mr. G. B. Jones and Mr. V. A. Stephen, for their assistance with the estimations and care of the instruments, and to Mr. A. Packham who discreetly arranged for the collection of the thyroids. To our many friends who so readily and without question collected and despatched the glands to this laboratory we are all deeply grateful.

The author volunteered in October 1955 to undertake this investigation as a part of the Operation Buffalo Biological Programme that was conducted conjointly by the Agricultural Research Council and the Medical Research Council. The data reported in this paper were cleared for publication in November 1957.

The instruments—a 1-in. diameter well-crystal scintillation counter together with amplifying, analysing, scaling, and timing units—were provided on loan for the occasion by the Atomic Weapons Research Establishment.

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# CHOLINESTERASE AND THE SECRETION OF THE BRAIN HORMONE IN INSECTS

By J. Monro*

*Summary*

Growth and moulting in insects are stimulated by a hormone from neuro-secretory cells in the brain. In diapause these cells fail to secrete their hormone, and van der Kloot (1955) correlated this failure with the disappearance of cholinesterase and cholinergic substance from the brain. The experiments reported here show that eserine, which blocks the action of cholinesterase, will also retard the adult development of *Phalaenoides glycine* Lew. (Lepidoptera) if injected into the pupa before the brain has released its hormone. But in *Pieris rapae* L. and *Danaus plexippus* L. (Lepidoptera) the brain has usually secreted sufficient hormone before pupation and eserine does not delay adult development when it is injected into these pupae. Apparently cholinesterase is essential to the secretion of the brain hormone in non-diapausing insects, and by blocking it an artificial diapause may be induced.

## I. INTRODUCTION

The influence of the brain on the growth and moulting of insects has been reviewed recently by Wigglesworth (1954). Briefly, the processes of growth and moulting require for their initiation, and possibly also for their maintenance, a hormone from the thoracic glands. The thoracic glands are themselves stimulated to produce this hormone by another hormone produced in neurosecretory centres of the brain. The mechanisms within the brain which lead to the production of the brain hormone are still largely unknown. In non-diapausing larval insects the activity of the neurosecretory cells appears to depend on adequate feeding (Wigglesworth 1934; Bounhiol 1938). Just after a moult and before feeding, such larval brains are quiescent but potentially active. By contrast the brain of a diapausing insect is called refractory because it does not become active, although the body of the diapausing insect will allow activity in a potentially active brain which has been transplanted into it (Williams 1952, 1956). A refractory brain may be converted into a potentially active one by chilling the diapausing insect for a sufficient time (Williams 1952). Clearly from such evidence the next step is to investigate the differences between refractory and potentially active brains.

At the biochemical level one piece of evidence has been reported. Van der Kloot (1955) found that the concentration of cholinesterase and cholinergic substance in the brain of the moth *Platysamia cecropia* fell to a low level at the onset of pupal diapause, and the electrical activity of the brain disappeared. During chilling and the completion of diapause development the concentration of cholinergic substance in the brain increased, and when the pupa was warmed

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the titre of cholinesterase also rose to a value close to that in the brain of a caterpillar in the final instar. In the ganglia of the ventral nerve cord, the concentration of cholinesterase and cholinergic substance remained at about the same level throughout. Van der Kloot suggested that diapause arises from a lack of cholinesterase and cholinergic substance in the brain, and that both these substances are necessary to neurosecretory activity.

In order to apply van der Kloot's idea to larval diapause, such as that of the codlin moth, *Cydia pomonella* (Andrewartha 1952), it is necessary to postulate a local deficiency of cholinesterase within the brain. Diapausing *Cydia* larvae are able to perform coordinated movements in walking and spinning new cocoons. Their brains are presumably electrically active and contain a functioning cholinesterase system. Yet they remain in diapause. Perhaps in them the deficiency of cholinesterase is confined to the vicinity of the neurosecretory cells. It may be that the lack of cholinesterase throughout the rest of the brain in *Platysamia* is associated with a rather inactive pupal life in which the coordinating activity of the brain is not called upon.

Van der Kloot's observations suggest that cholinesterase may also be essential for the neurosecretory activity of non-diapausing insects. Wigglesworth (1934) suggested that the secretory activity of the brain in *Rhodnius* is stimulated by nervous impulses through the ventral nerve cord. More recently Bounhiol (1952*a*, 1952*b*) has reported that in *Bombyx* also, the brain secreted only if its nervous connections with the ventral cord were intact. Perhaps cholinesterase functions merely in transmitting nervous stimuli which trigger the neurosecretory cells of the brain. On the other hand, in the moths *Platysamia* (Williams 1952) and *Phalaenoides* (Monro 1956), the secretory activity of the brain seems to be independent of its connections to the ventral cord. If the secretion of the brain hormone in these moths depends on cholinesterase, the latter probably acts within the brain itself.

The experiments recorded here were made in an attempt to demonstrate the influence of cholinesterase on the secretory activity of the brain by injecting eserine sulphate, an inhibitor of cholinesterase, into newly moulted pupae. For the purpose, comparisons were drawn between pupae of two different categories (Williams 1952). Williams distinguished between three types of lepidopterous pupae:

(*a*) *Non-diapausing.*—Those in which the brain is inactive while the thoracic glands go on secreting. Apparently the continued secretion of the thoracic glands is due to the presence of brain hormone carried over from the final larval instar (Bounhiol 1952*a*, 1952*b*).

(*b*) *Non-diapausing.*—Those in which the continued activity of the thoracic glands depends on further secretion by the brain soon after pupation.

(*c*) *Diapausing.*—Those in which both brain and thoracic glands are inactive for a long period after pupation.

If eserine suppresses or delays the secretory activity of the brain this could be shown by injecting it into a pupa of type (*b*) before the brain has secreted its

hormone and looking for a delay in adult development. In non-diapausing generations of *Phalaenoides glycine* Lew. the larvae pupate several days before the brain releases sufficient hormone to induce adult development (Monro 1956, 1957). This species therefore belongs to type (*b*). When eserine was injected into pupae of *Phalaenoides* a delay was observed but this may have been due to the temporary suppression of a stage of development after the brain had released its hormone (see Section III(*b*)). This could have been tested by injecting eserine into successively older batches of pupae. If the brain alone were influenced there would be a critical period corresponding to the time of release of the brain hormone. In pupae injected before this time there would probably be an increasing delay with later injections, and then no delay once the hormone was released. This would be true if eserine inhibits the release of the hormone, or if it returns the whole secretory cycle to its starting point. Unfortunately, sufficient numbers of *Phalaenoides* pupae were not available for such an experiment, so the possibility that eserine interfered with later stages of growth was tested on two other species of Lepidoptera, *Pieris rapae* L. and *Danaus plexippus* L. which belong to type (*a*) (see Sections III(*c*) and III(*d*)).

## II. MATERIAL AND METHODS

In South Australia, the noctuid *Phalaenoides glycine* has three generations a year. In summer the pupae usually develop into adults without entering diapause. The pupal stage is about 3 weeks at 27°C. For these experiments non-diapausing pupae were reared from final instar larvae taken from the field and kept at $27 \pm 1$°C in a photoperiod of 16 hr per day with grape-vine leaves for food.

In southern South Australia, *Pieris rapae* (Pieridae) has several non-diapausing generations and a winter diapause. The eggs of *Pieris* were collected in the field and the larvae reared on cabbage leaves at $27 \pm 1$°C in a photoperiod of 16 hr per day. Under these conditions no diapausing pupae were found among several hundred reared over two years.

*Danaus plexippus* (Danaidae) is a non-diapausing species which feeds on *Asclepias* spp. Larvae of *Danaus* were taken from the field to the laboratory during the final instar and fed on leaves of *Asclepias* at $27 \pm 1$°C in a 16 hr photoperiod per day until pupation.

All larvae were kept in colourless plastic dishes measuring 13·5 by 10 by 6·5 cm in a constant-temperature cabinet with white walls. Light was provided by an incandescent lamp (15-W, tungsten filament), attached to one wall of the cabinet. The length of the photoperiod was controlled by an electric "Venner" timing switch. The culture vessels were at a distance of 20–60 cm from the source of light. On pupation, the animals were transferred to glass tubes which were kept in a closed vessel over a saturated solution of potassium nitrate in water (93 per cent. R.H.). In the preliminary experiments, the ligatures were tied behind the head with silk thread. The parts in front of the ligature were cut away and the wound sealed with paraffin wax. When the brain of a pupa was removed (under $CO_2$ anaesthesia) the wound was covered with a window of glass sealed on with paraffin wax. Injections were made with standard hypodermic needles and an "Agla" micrometer syringe.

### III. Experiments and Results

#### (a) Preliminary Experiments

Preliminary experiments were performed on non-diapausing pupae of *Pieris* to find out whether adult development could proceed when the brain was removed just before or just after pupation. The animals were reared at $27 \pm 1$°C with a photoperiod of 16 hr (diapause-preventing) and assigned at random to one of two treatments. The controls were allowed to pupate normally, while in the other group the final instar larvae were ligatured behind the head at a stage indicated by the withdrawal of the ocellar pigment.

Table 1

INFLUENCE OF ESERINE SULPHATE ON THE DURATION OF THE PUPAL STAGE OF PHALAENOIDES GLYCINE AND DANAUS PLEXIPPUS

| Species | Group | Number of Animals | Mean Time of Development (days) | Range (days) |
|---------|-------|-------------------|--------------------------------|--------------|
| *P. glycine* | Controls | 6 | 16·2 | 14–21 |
| | Treated with eserine | 5 | 23·4 | 20–28* |
| *D. plexippus* | Controls | 13 | 8 | 7–9 |
| | Treated with eserine | 15 | 7·73 | 7–8† |

*Significantly different from controls ($P < 0.01$).
†Not significantly different from controls ($P = 0.3$–$0.4$).

Of 10 control animals all had developed black wing pigment in 4–5 days, while of the 10 which had been ligatured five died within 1–11 days of pupation, four developed black wing pigment in 10–11 days, and one showed no sign of adult development when dissected one month after pupation. A similar experiment in which the brain was removed during the first day after pupation, showed that it had ceased to influence the rate of adult development.

It would seem that the brain of *Pieris* has secreted sufficient hormone for the completion of adult development at, or about the time of, pupation. The newly moulted pupa of *Pieris* should, therefore, be a suitable subject for showing whether eserine can block steps in development later than the release of brain hormone.

Williams (1952) cited *Danaus plexippus* as an example of type (a). Pupae of this species were, therefore, used as well as those of *Pieris* to test the action of eserine on development subsequent to the release of brain hormone.

#### (b) *Influence of Eserine Sulphate on the Duration of the Pupal Stage in* Phalaenoides glycine

Non-diapausing pupae of *Phalaenoides* were assigned at random to one of two groups on the first day of this instar. In one group 0·01 ml of 0·1M eserine sulphate in physiological saline solution was injected into the thorax of each pupa.

The pupae of the other group each received an injection of 0·01 ml of unmodified saline solution. Both groups were kept at 27 ⊥1°C and 93 per cent. R.H. (saturated $KNO_3$) and inspected daily. The date on which orange and black pigment appeared in the wings was recorded. The behaviour of the moths which emerged was also observed.

The times required for adult development by each group are compared in Table 1. There was a significant delay in that group which received an injection of eserine compared with the group used as a control. The simplest explanation of this result seems to be that the release of brain hormone was delayed in the presence of eserine; the eserine was then slowly destroyed in the body, and the brain again
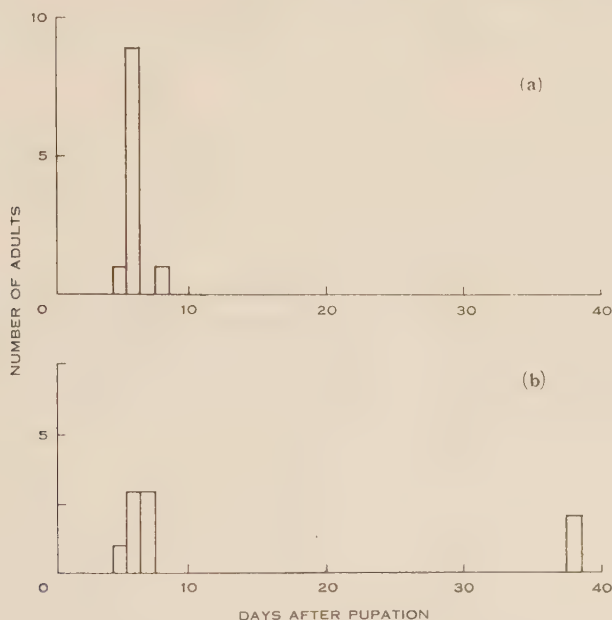


Fig. 1.—Duration of adult development from pupation to the appearance of wing pigment in *Pieris rapae*. (a) Pupae injected with 0·01 ml of saline solution during the first day after pupation; (b) Pupae injected with 0·01 ml of 0·1M eserine sulphate in saline solution during the first day after pupation.

became competent to secrete the hormone. Certainly the eserine appeared to be destroyed, or its effectiveness was reduced, because moths which emerged were able to walk or fly in a coordinated way.

(c) *Influence of Eserine Sulphate on the Duration of the Pupal Stage in* Pieris rapae

On the day of pupation pupae were assigned at random to two groups. In one group the pupae were given an injection of 0·01 ml of 0·1M eserine sulphate in physiological saline solution. Pupae in the other group received 0·01 ml of unmodified saline solution to serve as controls. Both sets of pupae were kept at 27 ⊥1°C and 93 per cent. R.H. (saturated $KNO_3$), and observed daily until the adults emerged, or until death. The time that the pupae in each group took to produce black wing

pigment was taken as the period needed for the development of the adult, because those treated with eserine were unable to emerge from the old pupal skin.

The periods needed for adult development are set out in Figure 1. Seven of the pupae that were treated with eserine reached the adult stage between the sixth and eighth day but two were delayed until the 38th day. It seems reasonable to exclude these two because a delay would be expected if, by chance, the eserine had been injected before the brain had secreted sufficient hormone to ensure the development of the adult. If these two are excluded the mean duration of adult development (up to the appearance of wing pigment) was 6·3 days for the seven treated pupae compared with 6·1 days for the 10 controls. The difference is non-significant.

### (d) Influence of Eserine Sulphate on the Duration of the Pupal Stage in Danaus plexippus

Pupae of *Danaus* were assigned at random to one of two groups within 24 hr of pupation. In one group each pupa received 0·02 ml of 0·1M eserine sulphate in physiological saline solution and in the other 0·02 ml of unmodified saline solution. The pupae were inspected daily and the date of emergence of adults recorded. In *Danaus* the pupal cuticle was shed even by those treated with eserine.

The times required for development from pupation to emergence are set out in Table 1. There was no significant difference between the two groups ($P = 0.3 - 0.4$). The pupae treated with eserine gave rise to adults which were unable to walk or fly though a large proportion of them emerged and expanded their wings. As in *Pieris* these adults showed irregular twitching of the legs.

### IV. Discussion

Eserine delayed development in *Phalaenoides* probably by inhibiting neurosecretory activity in the brain. In *Pieris* and *Danaus* pupae there was no delay if the brain had already secreted sufficient hormone and this will probably be found true of *Phalaenoides* also. The two *Pieris* pupae that were delayed by eserine probably had an insufficient titre of brain hormone in the blood at the time of injection. Later, the concentration of eserine was reduced, or the brain became less sensitive to it and was able to secrete once more.

Eserine not only inhibited the secretion of the brain hormone but it also interfered with the functioning of the voluntary muscles. The insects were not able to walk or fly and they showed an uncoordinated twitching of the legs. None of the *Pieris* was able to emerge and all those that were dissected out of the pupal cases showed these symptoms. But some of the *Phalaenoides* emerged and were able to walk and fly normally. The *Pieris* usually completed adult development within 5–8 days but the *Phalaenoides* took from 20–28 days. This suggests that the eserine gradually became inactive and that after 3 or 4 weeks the concentration had been reduced to the level at which it no longer interfered with the proper functioning of the voluntary muscles.

In *Pieris* either the muscles or nervous system were abnormal because they had differentiated in the presence of eserine, or eserine was still present. In dissections

of the thorax and legs no abnormality in the muscles was apparent at low magnification but this matter was not followed further. It is noteworthy that active cholinesterase does not seem to be necessary to the differentiation of thoracic muscles, though the extirpation of thoracic nerves and ganglia will prevent such differentiation in *Lymantria* (Kopec 1923), in *Telea* (Nuesch 1952), and in *Platysamia* (Williams and Schneiderman 1952).

An attempt was made to increase the proportion of *Pieris* pupae with an artificially induced diapause. Because the brain has secreted sufficient hormone for adult development at about the time of pupation, it ought to be possible to increase the proportion of pupae with delayed development by injecting eserine into final instar caterpillars nearing pupation. This was done but the experiment failed because all the eserine-treated animals died within the old larval skin at, or soon after, pupation.

The experiments on *Phalaenoides* and *Pieris* show that an artificial diapause may be induced by inhibiting cholinesterase. This result might have been predicted from van der Kloot's hypothesis and suggests that cholinesterase also plays an important part in the secretory activity of the brain in non-diapausing larvae and pupae. Possibly cholinesterase acts through its influence on nervous conduction between centres within the brain. In any event it is probably acting within the "inner mass" of the brain which, in *Platysamia*, is able to secrete the brain hormone when cut out of the brain of an adequately chilled pupa and implanted into a brainless pupa in diapause (Williams 1948). The inferred ativity of a large part of the brain in diapausing *Cydia* larvae is consistent with this hypothesis.

Williams (1952) described two sorts of non-diapausing pupae; one of which, like *Pieris* and *Danaus* had an inactive brain after pupation but still developed into an adult; while the other, like *Phalaenoides*, had an active brain in the pupal stage, and was unable to develop into an adult if the brain was removed. The second type of pupa may be converted into a "permanent" pupa by removing the brain just after pupation. Such "permanent" pupae are useful for testing the activity of implanted brains but in order to discover whether a pupa is of suitable type it has been necessary to remove the brain. The injection of eserine after pupation may possibly be used instead, as it is easier, and the subsequent mortality is much lower. Pupae which resemble *Pieris* in having already secreted sufficient brain hormone should develop into adults with little or no delay. Delay would indicate that the brain secreted its hormone after pupation, as in *Phalaenoides*. Other inhibitors of cholinesterase might also be useful, provided that they do not interfere with stages of growth which follow release of the brain hormone.

Williams (1951) has already described the action of pilocarpine in permanently preventing the development of diapausing pupae of *Platysamia*. He ascribed this action to the blocking of cytochrome synthesis but it may be simpler to regard it as due to the blocking of cholinesterase in the brain. Such an interpretation is strengthened by Williams' observation that pilocarpine was ineffective after the brain had secreted its hormone unless much larger quantities were injected.

## V. Acknowledgments

## VI. References

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# EFFECTS OF VARIOUS SUBSTANCES ON GROWTH OF SILKWORM TISSUES *IN VITRO*

By T. D. C. Grace*

*Summary*

Cells from the ovaries of late final instar silkworms have undergone mitosis in hanging-drop preparations for 19 days and survived for 29 days in a medium consisting of Wyatt's solution plus 10 components of the vitamin B complex, cholesterol, and extracts of endocrine and ovarian tissues. Attempts to subculture the cells met with only partial success.

The addition to Wyatt's medium of 10 members of the vitamin B complex and *meso*inositol improved the appearance of the cells but did not affect their growth or survival. Neither cholesterol nor nucleic acids had any effect on the growth or survival of the tissues.

The replacement of the sugars of Wyatt's medium by trehalose had a deleterious effect on growth and survival. When either glucose and fructose or sucrose were replaced by trehalose growth and survival were not affected. The addition to the medium of extracts of endocrine organs or of ovaries increased markedly the number of mitoses in the cultures.

Only ovaries from larvae which had reached the fourth instar produced cells which grew in culture. The only cells of the silkworm which grew in culture were those of the ovariole covering. Cell density within the cultures had an effect on their growth and survival.

## I. Introduction

In recent years several attempts have been made to grow insect tissues *in vitro* for long periods. The majority of these investigations have been concerned with developing the technique for use in the study of problems associated with the transmission of plant and animal virus diseases by insects (Trager 1935; Maramorosch 1956; Wyatt 1956).

The major difficulty has been the lack of balanced physiological solutions formulated from adequate knowledge of the composition of insect haemolymph. Although there are a number of "Ringer" solutions (see Roeder 1953, p. 161) for a variety of insects, these have been found in most cases to bear little relation to the composition of the haemolymph of the insects for which they were designed. Most of these solutions were developed for the purpose of maintaining tissues for short periods only.

In 1956, Wyatt, Loughheed, and Wyatt made chemical analyses of the haemolymph of the silkworm *Bombyx mori* (L.). From this information Wyatt (1956) formulated a medium in which she was able to obtain growth of ovarian tissue of

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the silkworm for 2 weeks in roller tube cultures. No attempts were made to sub-culture the tissues, and continuous growth of any insect tissue in culture has still not been achieved.

In the present investigations, attempts have been made to improve the growth of the cultures by studying (1) the effect of adding to Wyatt's medium various substances which may promote growth of the tissues, and (2) the effect of age and other characteristics of the tissues.

## II. Material and Methods

### (a) Insect Culture

The silkworms (*B. mori*) were reared in shallow metal trays and fed on mulberry leaves. During the winter months they were kept in incubators at a temperature of $24 \pm 2°C$. During the warmer months the trays were placed on benches in the laboratory.

As it is not possible to obtain freshly picked leaves during the winter, the silkworms were fed during these months on leaves stored in plastic bags and kept in a refrigerator at about 4°C. Under these conditions the leaves kept remarkably fresh for approximately 3 months, provided no water was present on the surface of the leaves.

### (b) Materials Used

(i) *Ovaries.*—In the majority of experiments the tissues used were from last stage (5th instar) larvae which had ceased feeding and in which the rosy coloration of the prepupae had appeared. Tissues from younger larvae were also cultured. No trouble was experienced with fungal, viral, or bacterial diseases of the silkworms. The larvae were surface-sterilized by washing twice with 70 per cent. alcohol, the second wash being a few minutes prior to dissection. No contamination was experienced using this technique. The larvae were starved for 24 hr to ensure an empty gut at the time of dissection.

Ovaries were obtained by removing the tergite from the 5th abdominal segment. Usually the ovaries remained in the larvae but were occasionally removed with the tergite. They were placed in a drop of medium and freed from any adhering fat-body or other tissue. They were then cut into pieces about 1 mm³. One ovary produced three to six pieces of tissue, depending on its size.

The ovariole sheaths were removed as follows: The ovarioles were separated from the ovarian sac; an ovariole was held by the oviduct with a pair of fine forceps; another pair of forceps was placed so that they straddled the ovariole. By gently pulling on the oviduct, the sheath was slipped off unbroken. It was picked up in a wide-mouthed pipette and cultured in a drop of medium.

(ii) *Haemolymph.*—Haemolymph was obtained by snipping off the caudal horn on the 8th abdominal segment and collecting it in chilled, sterile centrifuge tubes. It was essential to collect the haemolymph in chilled tubes to prevent the formation of melanin-like pigments which render it toxic to the tissues. The haemolymph was either centrifuged immediately or deep-frozen until required. Usually it was obtained from late 4th instar larvae, but occasionally from prepupae.

(iii) *Haemocytes.*—To obtain haemocytes for culturing, 5th instar larvae were bled from the caudal horn, the haemolymph being collected in a sterile centrifuge tube packed in ice. It was necessary to bleed about five larvae to accumulate enough cells for each culture. As soon as possible after bleeding, the haemolymph was lightly centrifuged to collect the cells into a firm pellet which could be cut into pieces about 1 mm³.

(iv) *Embryos.*—Embryos at two stages of development were studied. The youngest embryos were those in which segmentation was just completed but the appendages were still small outgrowths. The older embryos were nearly fully developed, the mandibles and eyes were pigmented, and the heart and gut showed movements.

By careful manipulation it was possible to remove the embryos from the eggs without breaking them, and without carrying over much yolk. Before being set up in culture the embryos were washed three times in the medium. The hypodermis of the older embryos were torn into several pieces to ensure that all the tissues were bathed by the medium. Although it was very easy to transfer the older embryos to a hanging drop without breaking them, the younger embryos invariably broke apart when being lifted through the surface film of the medium.

## (c) Basic Medium

In all the experiments described in this paper Wyatt's (1956) physiological solution containing 2 per cent. heat-treated haemolymph (60°C for 5 min) was used as the basic medium. Media were sterilized by passage through a Seitz or sintered-glass filter. Penicillin and streptomycin were routinely added to the media at concentrations of 0·03 and 0·1 mg/ml respectively.

## (d) Tissue Extracts

(i) *Endocrine Organs.*—Ring glands were taken from 200 larvae of *Lucilia cuprina* (Wied.) in the middle of the last instar, and the prothoracic glands from 20 last-stage nymphs of *Periplaneta americana* (L.). Adhering tissues were removed, and the glands transferred to 1·0 ml of medium (which did not contain any haemolymph) in a sterile centrifuge tube and macerated. The resulting suspension was stirred thoroughly and allowed to stand for 1 hr. It was then centrifuged for 5 min at 2200 g. The supernatant was filtered through a sintered-glass filter and a solution containing 2 per cent. heat-treated haemolymph added.

(ii) *Silkworm Embryo.*—Embryo extract was obtained from eggs in which diapause had been broken by immersing them (24 hr after laying) in concentrated (35 per cent.) hydrochloric acid for 3 min. The eggs were washed several times in tap water and dried on filter paper. After the acid treatment the eggs were incubated for 6 days at 25°C, then surface-sterilized in 70 per cent. alcohol, and finally ground in a mortar with a little medium to which a small amount of sterile sand had been added. The suspension was then centrifuged at 90 g for 5 min to remove the tissue debris and sand. The supernatant was transferred to a fresh sterile tube and centrifuged at 9000 g for 60 min. The final supernatant which was still slightly

cloudy was then transferred to a sterile tube, stoppered, and kept in a deep-freeze unit until required. (It was not possible to clarify the extract by centrifugation at this speed.)

By placing the extract in boiling water for 2 min a large amount of protein was precipitated. Centrifuging now produced a clear supernatant.

### (e) *Techniques*

Glassware was cleaned by boiling with soft soap for 10 min, rinsing in tap water, and re-boiling for 10 min in distilled water. It was then rinsed once in distilled water, dried, and sterilized in an oven at 160°C for $1\frac{1}{2}$ hr.

The principal culture method used was the hanging drop in small depression slides. Single explants were placed in approximately 0·005 ml of medium. Cultures were also set up in Carrel flasks, roller tubes, hanging drops containing perforated "Cellophane", and solid clots of fowl plasma.

The criteria used to estimate growth and survival were respectively the presence of mitoses in cells which had migrated from the tissues 3–4 days after setting up the cultures, and lack of granules, fatty droplets, or other abnormalities of the cytoplasm.

## III. Experimental

### (a) *Cell Growth in the Basic Medium*

In previous experiments carried out over a period of $2\frac{1}{2}$ years all the substances (vitamins, cholesterol, nucleic acids, hormone, and tissue extracts) studied had been tested for their effect on growth of silkworm larval tissues in Trager's (1935) medium. However, little or no effect was noticed when these substances were present, perhaps because the medium did not resemble sufficiently the haemolymph of the silkworm. Eventually, in Trager's medium to which had been added B vitamins, cholesterol, and 10 per cent. silkworm plasma it proved possible to keep the cells active for 5–6 days, but very few mitoses occurred after 3 days and the tissues never survived for longer than 9–10 days.

In all experiments with Wyatt's medium it was noted that, during the first 24 hr after setting up the cultures, very few cells migrated from the tissues, and those that did stayed close to them. During the next 24 hr the number of cells which moved away from the tissues increased considerably. The majority of cells adhered to the cover-glass or the surface of the drop although some remained suspended in the medium. The cells which adhered to the surfaces became either irregular in outline or spindle-shaped and moved in an amoeboid manner, whereas those which were in suspension remained spherical. After 72 hr many cells had migrated well away from the explant. Although they remained active, very few cells migrated further so that, after a few days, a number of cells formed a "ring" around the explant. The cells within the ring continued to move in all directions but, as the culture aged, the number which came to lie near the explant increased. It was also observed many times that the number of mitotic divisions increased after the first 48–72 hr, continued at a constant level for a few days, and then gradually dropped as more and more cells became granulated. Eventually the

mitoses ceased even though there were a large number of active, transparent cells near the explant. Finally, granulation and subsequent degeneration began in the cells furthest from the tissue, and gradually moved towards the explant until all cells had become degenerate. Frequent renewal of the medium did not prevent degeneration of the cells.

### (b) Growth in Modifications of the Basic Medium

The basic medium has been modified by addition of a number of metabolites or extracts. These together with their effects are listed in Table 1.

(i) *Vitamins.*—The members of the vitamin B complex are necessary for normal growth of insects (see Trager 1953). The following members of the B group were added: thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, biotin, folic acid, *p*-aminobenzoic acid, choline, and *meso*inositol. At a concentration of 10 μg/ml

TABLE 1
EFFECT ON TISSUES OF ADDITIONS TO WYATT'S MEDIUM
+ = better growth or survival;  — = harmful;  0 = no effect

| Modification | Effect | Modification | Effect |
|---|---|---|---|
| B vitamins (10 μg/ml) | — | Trehalose replacing either hexoses or sucrose | 0 |
| B vitamins (0·01 μg/ml) | 0 | Endocrine extract | + |
| *meso*Inositol | 0 | Embryo extract | — |
| Cholesterol | 0 | Treated embryo extract | 0 |
| Ribonucleic acid, thymus nucleic acid (separately or together) | 0 | Ovary extract | + |
| Trehalose replacing all sugars | — | Endocrine extract plus trehalose replacing all sugars | + + |

(except choline at 100 μg/ml, and *meso*inositol at 2 μg/ml) heavy granulation of the cells occurred after 4 days, the cytoplasm became threadlike, and the cells did not survive longer than 1 week. When only *meso*inositol at 2 μg/ml or all vitamins at 0·01 μg/ml were added, there was a definite improvement in the appearance of the cultures. Many more cells migrated from the tissues but there was no increase in the number of mitoses or survival of the tissues.

(ii) *Cholesterol.*—This growth factor (essential for normal growth in the Diptera, Lepidoptera, and Coleoptera) was added to the basic medium at a concentration of 0·03 mg/ml to produce a saturated solution. Its presence did not appear to have any effect on the survival or growth of the tissues.

(iii) *Yeast Nucleic Acid and Thymus Nucleic Acid.*—These nucleic acids were added separately or together to the medium in several concentrations (10, 20, and 30 μg/ml). In no instance did the addition of either or both of these acids have any effect.

(iv) *Trehalose.*—Wyatt and Kalf (1956) have recently shown that trehalose is present in silkworm blood at the surprisingly high concentration of 500 mg/100 ml.

Three sugars (glucose, fructose, and sucrose) were included in Wyatt's medium. In media containing trehalose as the only sugar at the same concentration (150 mg/100 ml) as the sugars in the basic medium, or at 500 mg/100 ml, there was very little migration of cells from the tissues even after 1 week in culture.

When either the glucose and fructose or the sucrose were replaced by trehalose the number of cells migrating from the tissues was about the same as in the original medium. However, when trehalose was used in the medium containing extracts of endocrine organs (see below) good cell growth and multiplication was obtained.

(v) *Endocrine Organ Extracts.*—The number of cells which migrated from the tissues in the hormone-containing medium (prepared as described in Section II) was the greatest ever obtained (Plate 1, Fig. 1). Mitoses were first noticed 8 days after establishing the cultures, and cells in all parts of the medium underwent mitosis, whereas previously the mitoses were found to be more numerous in cells near the tissues. The period from metaphase (the first easily recognizable stage of mitosis) until complete cell division was 15–20 min. During the next 5 days numerous mitotic figures were noticed both in cells near the explant and at the periphery of migration. The number sharply decreased until, by the 17th day, only one or two near the explant could be found. No granulation or decreased mobility of the cells was observed in any of the cultures for about 14–16 days. As found by other workers, the first signs of granulation were in the cells furthest from the tissue. By 21 days the majority of cells had become round and granular but a few cells close to the explant were still transparent and active.

(vi) *Embryo Extracts.*—The supernatant from ground, centrifuged eggs, when added to the medium at concentrations of 2–5 per cent. caused heavy granulation of the cells within 4 days.

In media containing 5 or 10 per cent. of the treated embryo extract (see Section II), large numbers of cells migrated from the tissues. All the cells were still transparent after 9 days and active except for a few at the margin of migration. Granulation of the cells developed subsequently until, by 14 days after the cultures had been prepared, only a few transparent cells were present.

(vii) *Ovarian Extracts.*—The fact that the cells nearest the tissue seemed to survive longer than those further away suggested a possible effect by the tissue on the medium. Twelve ovaries from late, last-instar larvae were ground very thoroughly in 1·0 ml of haemolymph-free medium. The solution was filtered and 2 per cent. of heat-treated blood (60°C for 5 min) was added.

Tissues set up in this medium showed very marked migration of the cells with numerous mitoses after 48 hr, comparable with that obtained using the medium containing hormone extract. Granulation of the cells began to appear about the 14th day, and by the end of 21 days the majority of the cells were degenerating. Apparently the ovarian extract had some beneficial effect for, prior to the use of this medium and that containing hormone extracts, mitoses were very few and only rarely did cells survive longer than about 2 weeks.

As the presence of either endocrine organ or ovarian extracts in the medium had a very beneficial effect on growth, a medium was prepared in which both extracts

were present in the same amounts as when they were added singly. Migration and multiplication of the cells was profuse. Mitoses were first noticed 48 hr after establishing the cultures and were numerous for 12–14 days. During this time no granulation occurred and the cells were active. After the 14th day the number of mitoses decreased and about the 16th day some of the outermost cells contained granules. Mitoses could still be found near the explant after 18 days but they were scarce. Granulation of the cells spread progressively inwards and by the 20th day no mitoses were found and the majority of the cells were degenerating.

An attempt was made to test further the effect of the endocrine organ and ovarian extracts on growth. In a previous experiment in which sucrose, fructose, and glucose were replaced by trehalose, the growth and multiplication of the cultures was very poor. This experiment was repeated, with the addition of endocrine organ extract to the medium. After about 3 days migration was marked. On the 8th day ovarian extract was added when changing the medium. Quite a large number of mitoses were observed 2 days later and one or two could still be found after 19 days. A number of cells in these cultures had quite obviously undergone mitosis to the stage where cell division was almost but not quite complete (Plate 1, Fig. 2). By the end of 21 days granulation was far advanced. The results of this experiment demonstrate that the endocrine organ and ovarian extracts have a beneficial effect on the survival and growth of the tissues.

### (c) The Effect of "Conditioned" Medium

To determine whether conditioning of the medium had any effect on growth of insect tissue, six pieces of ovary tissue were placed in 0·1 ml of medium and incubated for 48 hr at 26°C. The medium was then used in hanging-drop cultures each containing one explant in 0·005 ml of medium. The migration of cells was poor and many became granulated after 4 days. It is possible that the practice of changing the medium in hanging-drop cultures every 48 hr may have an adverse effect on growth. In some cultures the medium was, therefore, changed only every 4th day and in others once weekly. In neither series of cultures was survival or growth increased.

### (d) Modification of the Vapour Phase

In an attempt to study the effects of modification of the gas phase either $CO_2$ or $O_2$ was blown under the coverslip for 30 sec prior to sealing it to the slide. Each time the medium was changed the gassing procedure was repeated. Control cultures which were not treated in any way were set up at the same time. The migration of cells from the tissues and the length of survival were the same as in the control cultures when either $CO_2$ or $O_2$ was used. The pH of the medium dropped from 6·4 to 6·2 after 48 hr in those cultures gassed with $CO_2$.

To decrease the $CO_2$ tension in normal cultures, two very fine capillaries were sealed with paraffin on to a depression slide so that there could be a flow of gas in and out of the chamber. Drying out of the cultures did not occur. These treatments had no effect on the growth or survival of the tissues.

### (e) *Modifications of the Tissue Explants*

In all the studies on insect tissue culture in which the silkworm has been used, cells from the ovaries are the only ones which have grown in culture (Trager 1935; Grace 1954; Wyatt 1956). The fat-body, gut, salivary glands, muscles, testes, nerve, blood cells, and hypodermis have all failed to grow.

In this section experiments will be described in which the effects on growth have been studied of (i) the age of the ovaries used, (ii) culturing the ovariole sheath removed from the ovariole, (iii) increasing the cell concentration in the cultures, (iv) other tissues, particularly embryonic tissues and haemocytes, (v) subculturing.

(i) *Age of the Ovaries.*—The ovaries of 2nd, 3rd, 4th, and 5th instar larvae (early and later in the instar) were cultured in Wyatt's basic medium to which was added B vitamins, cholesterol, and 10 per cent. silkworm larval plasma.

TABLE 2

EFFECT OF AGE OF THE OVARIES ON GROWTH AND SURVIVAL

| Instar from which Ovaries Taken | No. of Explants per Culture | Growth and Survival |
|---|---|---|
| 2nd or 3rd instar | 2 ovaries | No growth. Survived for 6 days. No cell migration |
| 4th instar | 2 ovaries | No growth. Survived for 10 days. A few cells migrated into medium |
| 5th instar | | |
| (*a*) Early | 2 explants | No growth. Survived for 16 days. Fair cell migration |
| (*b*) Late | 1 explant | Limited growth. Survived for 16 days. Good migration of cells into medium |

The ovaries of the early instar larvae were too small to cut into pieces so the ovarian sac was opened and the ovarioles were teased out. Usually (because of their small size) two ovaries were placed in each hanging-drop culture. The ovaries of the 5th instar larvae were cut into three to five pieces depending on their size and one explant placed in each culture.

The results as set out in Table 2 show that, at least on this rather unsatisfactory medium, no growth and very short survival was obtained in the cultures of 2nd, 3rd, and 4th instar ovaries. In cultures of the ovaries from 5th instar larvae no difference was observed in the length of survival between those taken early or late in the instar, but the amount of cell migration and the number of mitoses observed were much greater in cultures of the late 5th instar ovaries. After about 5 days in culture some of the ovarioles of the late instar ovaries showed movements, which were due to the contractions of the muscle sheath around the ovariole. Muscular contractions of the tissues have not been observed in any larvae but only in pupae at least 3 days after pupation.

(ii) *Cultures of the Ovariole Sheath.*—In order to determine whether the only cells which grow in culture are those of the lining of the ovariole, the sheaths were

removed from the ovarioles of the prepupae (as described above) and cultured in hanging drops. The naked ovarioles were also cultured. Large numbers of cells migrated from the sheaths and mitoses were observed over a period of about 5 days. After 10 days in culture many of the cells were granulated and the cultures were discarded after 2 weeks. Not one cell migrated from the naked ovarioles.

(iii) *Size of Explant.*—It had been observed that, in cultures containing either the endocrine organ or the ovarian extract or both, cell migration and multiplication were very marked. However, although the period of survival was somewhat increased, the tissues were still not capable of surviving and multiplying for long periods. It is possible that the better growth in the cultures is not only due to the presence of extracts but also to a second factor, namely the size of the population of cells in the medium. To test this, a series of cultures was set up in which an explant was placed in a drop of medium containing hormone extract. After 9 days, when the cell population was dense and a few mitoses could be found near the tissue, an explant from another culture was added. Twenty-four hours later mitoses were very numerous. Dividing cells were found near the explant, and there were also many at the periphery. The majority of cells rounded up and the cytoplasm became dense prior to division, but mitoses were observed in some cells (especially those far from the explant) in which cytoplasm remained transparent and spread in a thin sheet across the coverslip. Five days after adding the second explant the mitoses became scarcer and by the 7th day all divisions had stopped. Although a very large number of cells were still healthy, many of the outer ones had become granulated. After 25 days granulation of the cells had increased and the cultures had degenerated by the 29th day.

The control cultures, containing only one piece of tissue, showed a few mitoses until they were 14 days old, after which the cells began to degenerate.

(iv) *Culture of Tissues from Embryos.*—A few attempts were made to grow embryonic tissues but have so far met with little success. The younger embryos failed to survive in culture longer than 10 days. A large number of cells containing what appeared to be fat droplets were liberated into the medium, but no mitoses were observed. The gut and heart of the older embryos continued to contract for up to 14 days but there was no growth of cells into the medium and no mitoses were observed.

(v) *Haemocyte Cultures.*—Haemocytes were placed in hanging-drop cultures in Wyatt's medium containing 10 members of the vitamin B complex, endocrine organ extract, and 2 per cent. heat-treated haemolymph. Even when a crystal of phenylthiourea was added, all cells had turned black within 24 hr and were degenerating after 3 days.

(vi) *Attempts to Subculture.*—During the early stages of the work several attempts were made to subculture the tissues. The method consisted of removing the explants from the culture, care being taken to leave behind as many free cells as possible. After renewing the medium, the tissues were implanted in one hanging drop and the cells in another.

It was not until tissue extracts were added to the medium and the consequent increased population of cells was obtained that it was possible to obtain a successful

subculture. After 14 hr the subculturing cells had begun to migrate from the tissues and migration and multiplication continued for about 3 days, but the cell population in the medium never reached the size of the parent cultures.

In several cultures, when the cells were quite numerous and mitoses were still evident, a second subculture was made, separating the cells from the tissues. Again cells were present after about 24 hr and one or two mitoses could be found. The number of cells increased very slowly over the next 2–3 days and by the 5th day there would usually be about 300 cells in the medium. The number of cells did not increase any further and granulation started about the 6th day, the cultures not surviving for more than 8 days. It was never possible to re-implant the inoculum more than twice or to subculture the cells more than once.

## IV. Discussion

In the experiments outlined above three facts stand out. First, it is obvious that, although Wyatt's medium is superior for the *in vitro* culture of silkworm ovaries to any other medium which has previously been used, it does not contain all the factors necessary for the continued growth of the tissues.

Second, after the cells have been in culture for about 16 days, the number of mitoses decreases sharply and many of the cells begin to degenerate. Only in a few cultures have the cells stayed healthy for as long as 3–4 weeks.

Third, with the addition of the endocrine organ and ovarian extracts the number of mitoses and the number of cells present in the medium were greatly increased, but the length of survival and the periods during which mitoses are present have not been increased much beyond that obtained by Wyatt (1956).

The partial success obtained when the tissues were subcultured indicated that the medium contained nearly all the factors needed to prolong growth but that further detailed studies of the growth requirements of the tissues need to be carried out.

The presence of silkworm embryo extract had less effect than was anticipated on the basis of work with vertebrate cultures. Whole extract had a deleterious effect which could be ascribed to the large amount of yolk and protein material present. Gaulden and Kokomoor (1955) reported that "the frequency of mid mitotic neuroblasts in grasshopper embryos in hanging-drop cultures increases as the quantity of yolk in the cultures was increased up to quantity equivalent to one-quarter that in the egg". Although no yolk *per se* was present, the extract was made from 2000 eggs in 1·0 ml of medium. After boiling the extract, when much of the protein was removed, the tissues grew better. A similar result was obtained in a comparison between whole plasma and plasma in which much of the protein had been removed by heating. Apparently insect tissues (at least the ovaries) under culture conditions, do not tolerate a large amount of protein. In this they differ markedly from vertebrate tissues for which it is important to include proteins in the medium to obtain growth.

The improvement in growth resulting from the addition of cells to the medium is evidence that the larger the size of the inoculum (up to a limit) the more quickly

GROWTH OF SILKWORM TISSUES IN VITRO



0·05 MM
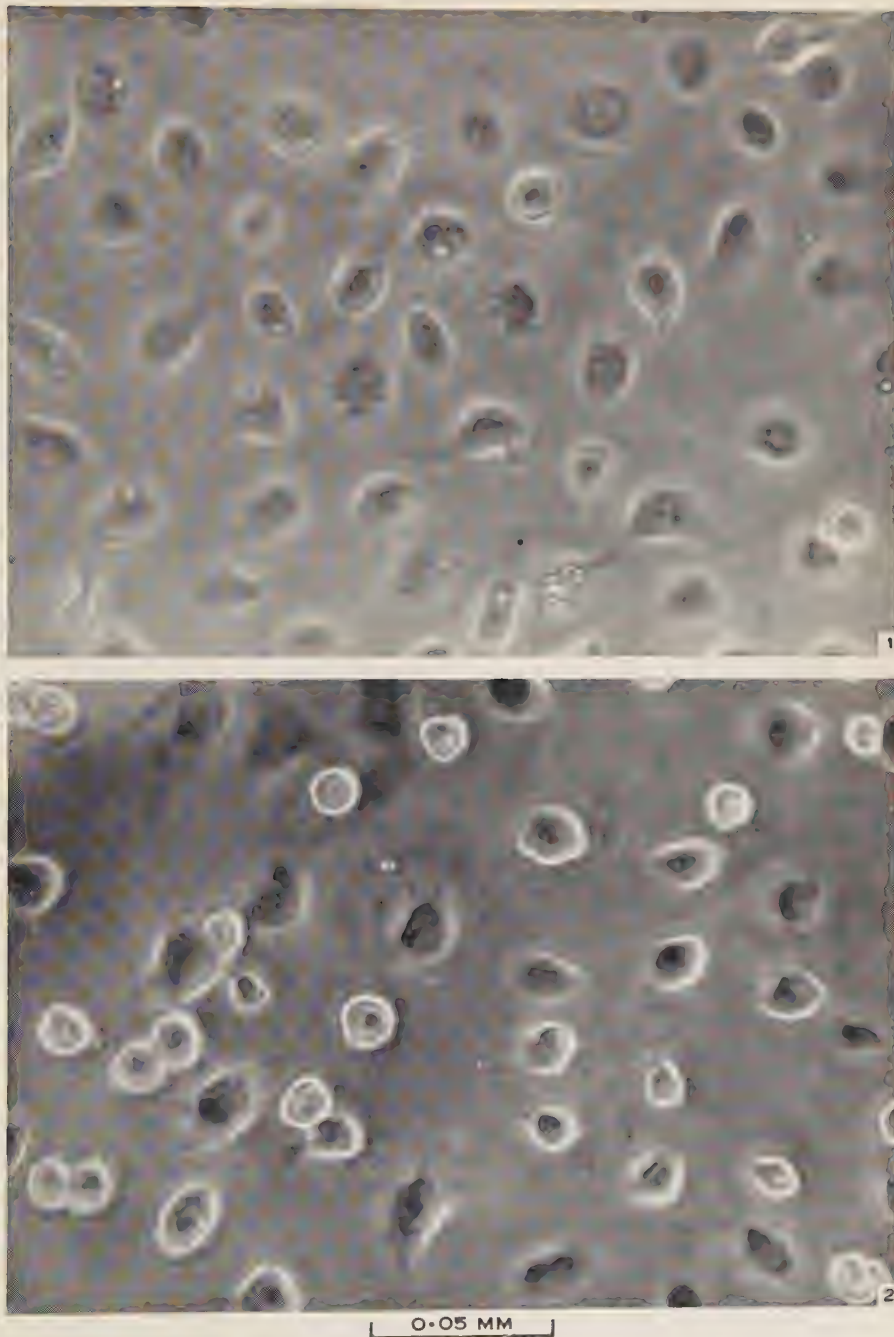
Fig. 1.— Cells from the lining of the ovarioles of 5th instar larvae of the silkworm cultured
for 8 days in Wyatt's medium containing hormone extract. × 380. Phase contrast.

Fig. 2. Numerous cells in telophase in Wyatt's medium containing hormone extract,
ovary extract, and trehalose, after 20 days. × 380. Phase contrast.

will growth proceed. In growth studies of bacteria and vertebrate tissues the size of the inoculum (either separate cells or explants) has been shown to determine to a large degree whether the cells will grow or not. Experiments such as these are very similar to those in which conditioned media were found to increase the ability of the cells to grow in culture. It is probable that the greater the size of the inoculum the less time is needed for the medium to become optimal for growth.

The results of the subculturing experiments are interesting in that they show (1) that only in those cultures in which the cell population was high was it possible to obtain growth after subculturing; and (2) that the presence of the explant has a growth-promoting effect on the cells. In experiments using the ovaries of diapausing pupal *Callosamia promethea* (Drury) it has been possible to carry the cells through a second subculture only if a piece of surviving ovary was present (Grace, unpublished data). The results are encouraging when it is recognized that it is difficult to maintain growth in first subcultures of some vertebrate tissues, but after they have grown in the first subculture it becomes relatively easy to make subsequent subcultures. Until it is possible to carry out repeated subcultures, the continuous growth of insect tissues cannot be said to have been achieved.

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# THE MUTAGENICITY OF FORMALDEHYDE MEDIUM TO *DROSOPHILA MELANOGASTER* LARVAE

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*Summary*

There is a linear decrease in the mutagenicity of formaldehyde medium with aging, whether it is preworked or not.

There is little or no decrease in the concentration of formaldehyde in the medium from preparation to 7 days later.

It is suggested that formaldehyde itself is the mutagen, but that it forms a reaction product with some food constituent, though presumably not with protein. This compound probably breaks down in the larvae, releasing mutagenically active formaldehyde.

## I. INTRODUCTION

Auerbach (1953), Auerbach and Moser (1953), and Herskowitz (1954) have based their ideas on the mutagenic action of formaldehyde medium on the expectation that the mutagenicity would be zero after 24 hr working by larvae. However, Barker and Davern (1956) showed that 0·1 per cent. formaldehyde medium (dead yeast fortified) remains mutagenic for at least 10 days, and that there is a linear decrease in the mutagenic response with increased preworking period. Further, they suggested that this decreased response with time may be merely an effect of aging of the food. This possibility has been tested and results are presented in this paper.

Additional evidence has been obtained by chemical estimation of the formaldehyde concentration in the medium at intervals after its preparation.

## II. MATERIALS AND METHODS

The experimental material was the Oregon-R-C stock of wild-type *Drosophila melanogaster*. All cultures were maintained at $25 \pm 1°C$, except when removed from the constant-temperature room for short periods for mating and inspection. The flies were kept on dead yeast fortified medium and all cultures were seeded with live yeast. The frequency of sex-linked recessive lethal mutations was used as an index of mutation rate. These were detected by use of the Muller-5 stock; $F_2$ vial cultures were scored as lethals when no wild-type males were present. Vials with less than 20 flies or 10 males were discarded from the score.

To prepare the formaldehyde medium, the measured amount of C.P. formaldehyde (36 per cent. w/v HCHO) was thoroughly stirred in when the boiled medium

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cooled to about 60°C. The amount added was calculated to give 0·1 per cent. formaldehyde as volume percentage of the liquid medium. The mutagenicity of this medium was determined after it had remained at 25°C for 0, 2, 4, 6, and 8 days. The procedure used was that of experiment 4 of Barker and Davern (1956).

For the chemical estimation, 0·1 per cent. formaldehyde medium was prepared. The first analysis was done as soon as the medium solidified. The remaining medium was maintained in $\frac{1}{4}$-pint culture bottles at 25°C, and the analysis was repeated daily for 7 days. Each day's estimation was duplicated (taking two bottles at random), using the following procedure. A sample of 1 g of medium was homogenized in 80 ml of distilled water. This suspension was divided into two 40-ml portions, of which one was made to 50 ml with distilled water and the other acidified to pH 1 with 2N sulphuric acid, then brought to 50 ml. Both solutions were then filtered. The non-acidified medium will give free formaldehyde and the acidified free formaldehyde plus formaldehyde bound to protein. Two 0·5-ml aliquots of each solution were taken and 5·0 ml chromotropic acid reagent (MacFadyen 1945) added to each. These were heated for 30 min on a boiling bath and the optical densities then read at wavelengths of 520, 575, and 615 mμ. These wavelengths were determined by deriving the absorption curve, where maximum absorption was observed at 575 mμ, while at 520 and 615 mμ absorption was one-half the maximum. A colour correction for irrelevant absorption was carried out on the optical densities at these wavelengths (Morton and Stubbs 1946). The corrected optical density (O.D.) was taken as $2 \times$(O.D. at 575 mμ)—(O.D. at 520 mμ+O.D. at 615 mμ). The corrected optical densities were converted to μg formaldehyde per ml of medium solution by comparison against readings from a stock formaldehyde solution. Preliminary tests of the estimation method showed that the recovery of formaldehyde is satisfactory. This was done by adding a known amount of formaldehyde solution to the media solution immediately before heating in the boiling bath.

## III. Results

The effects of aging fortified formaldehyde medium on the mutagenic response are shown in Table 1, where the results of Barker and Davern (1956) for preworked fortified formaldehyde medium are included for comparison. The values for angle response in the two missing plots were fitted by regression analysis.

Analysis of variance of the results (see Table 1) showed no significant differences between days or between treatments and the linear interaction term was not significant. The linear mean square between days is significant when tested against the theoretical variance, but is not when tested against the non-linear interaction as error. The regression coefficient ($b$) of mutagenic response on days is $-0·702$, where $b$ is the change in angle response per day. Since this is similar in magnitude to that found by Barker and Davern (1956), it is reasonable to infer that there is a negative regression of mutagenic response on days, but that it is the same whether the medium is worked or not.

The results of the chemical analyses of formaldehyde media are shown in Table 2. Each day the acidified and non-acidified media were compared using

samples derived from the one bottle. Since these samples, therefore, are not independent, it would be expected that the error associated with such comparisons is smaller than that associated with comparison of bottles. The analysis of variance therefore follows the split-plot form (Cochran and Cox 1957), the days differences

TABLE 1

LETHAL MUTATION FREQUENCIES INDUCED BY 0·1 PER CENT. FORMALDEHYDE WHERE THE MEDIUM AGED WITH AND WITHOUT LARVAL WORKING

| Treatment | Data | Period of Aging (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 |
| Medium worked by larvae* | No. of lethals/total tested | 27/228 | 7/274 | — | 16/503 | 24/817 | 23/781 |
| | Percentage lethals | 11·842 | 2·555 | — | 3·181 | 2·938 | 2·945 |
| | Angle response | 20·16 | 9·155 | (12·600) | 10·272 | 9·882 | 9·896 |
| Medium not worked by larvae | No. of lethals/total tested | 15/279 | 12/211 | 7/201 | 4/153 | 3/122 | — |
| | Percentage lethals | 5·38 | 5·69 | 3·48 | 2·61 | 2·46 | — |
| | Angle response | 13·39 | 13·80 | 10·72 | 9·26 | 8·97 | (7·214) |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Between days | (5) | |
| Linear | 1 | 69·016 |
| Remainder | 4 | 4·513 |
| Between treatments | 1 | 6·179 |
| Interaction | (3) | |
| Linear | 1 | 0·154 |
| Remainder | 2 | 16·832 |
| Theoretical variance† | ∞ | 2·30 |

*Results of this treatment are from Barker and Davern (1956).

†Theoretical variance = 820·7/$n$, where $n$ = average group size = 356·9.

being tested against one source of error and the media differences against another. Analysis of variance (see Table 2) shows that there are significant differences between bottles within days and between media, but that there is no difference between days. This suggests there is little or no loss of formaldehyde from the medium for

at least 8 days. However, there is considerable loss during preparation. The medium was prepared to contain 1083 $\mu$g formaldehyde per ml of media. This is equivalent to the 0·1 per cent. concentration used in the mutation tests. With a 1/100 dilution, the estimation solution will be expected to contain 10·83 $\mu$g per ml. The results in Table 2 show that an average of 40 per cent. of the added formaldehyde is lost during medium preparation.

TABLE 2

CHEMICAL ANALYSIS OF SAMPLES OF MEDIA TAKEN DAILY FOR 8 DAYS

Results given as $\mu$g formaldehyde/ml 1/100 media solution

| Medium | Bottle No. (two replications) | Days | | | | | | | |
|--------|------|------|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Acidified | 1 | 5·66 | — | 7·22 | 7·16 | 6·36 | 6·74 | 6·62 | 5·82 |
| | | 5·72 | — | 6·64 | 6·86 | 6·42 | 6·60 | 6·34 | 6·06 |
| | 2 | 7·10 | — | 7·46 | 7·08 | 6·64 | 6·24 | 6·88 | 6·76 |
| | | 7·00 | — | 7·38 | 6·90 | 6·58 | 6·42 | 7·38 | 7·24 |
| Non-acidified | 1 | 5·36 | — | 6·58 | 6·70 | 5·74 | 6·20 | 5·60 | 5·52 |
| | | 5·84 | — | 6·82 | 6·94 | 6·18 | 6·60 | 6·80 | 5·88 |
| | 2 | 6·74 | — | 7·16 | 6·80 | 6·00 | 6·40 | 6·20 | 6·64 |
| | | 6·72 | — | 7·42 | 6·82 | 5·88 | 6·18 | 6·60 | 6·68 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Mean Square | F |
|---------------------|--------------------|-------------|---|
| Between days | 6 | 0·8876 | 1·001 |
| Between bottles within days (error A) | 7 | 0·8868† | |
| Between media | 1 | 1·2244 | 18·439*** |
| Media × bottles within days | 7 | 0·0276 | |
| Media × days | 6 | 0·0540 | |
| Between replicates (error B) | 28 | 0·0664† | |

*** $P < 0.001$.   † $F = 13.355$***

The acidified solution contains significantly more formaldehyde than the non-acidified. However, the average difference (i.e. the protein-bound formaldehyde) amounts to only about 5 per cent. of the total. Thus there is very little loss of formaldehyde from the media with storage, even though only a small fraction is protein-bound. It is possible that some formaldehyde may be bound to food constituents other than proteins.

## IV. DISCUSSION

The results show two contrasting features. First, there is little or no decrease in the concentration of formaldehyde in the medium from preparation to 7 days later. Secondly, there is a linear decrease in the mutagenic effectiveness of the medium with aging, whether it is preworked or not. These results need to be related to ideas on the mode of action of formaldehyde.

There are four ways of mutagenic action of formaldehyde when taken into the body via the digestive tract of larvae:

(i) Free formaldehyde is the mutagen that is taken into the body and reaches the gonads as such. This is unlikely in view of the high reactivity of formaldehyde.

(ii) Formaldehyde is itself the mutagen but it forms a labile compound with some food constituent and is released either in the gonads or even in the gonadal cells themselves. Auerbach (1951) suggested this mode of action and further suggested that protein was the food constituent involved. The results of Alderson (1957) certainly indicate that formaldehyde is itself the effective mutagen.

(iii) Formaldehyde may react with a food constituent when it is added to the medium at about 60°C to produce the mutagen.

(iv) The mutagen may be formed by some reaction in the body either during digestion or in the germ cells themselves.

The second of the above hypotheses is the simplest and the most attractive. Auerbach (1952) showed that 0·3 per cent. formaldehyde solution injected into adult males gave rise to 3–4 per cent. sex-linked lethals, while Herskowitz (1955) obtained 2·2 per cent. sex-linked lethals following a sperm bath with 2·0 per cent. formaldehyde solution. By immersing pupae for 3 hr in a 10 per cent. aqueous solution of formaldehyde, Khishin (1956) obtained 1·33 per cent. sex-linked lethals. In these cases, formaldehyde itself is most likely to be the mutagen. On the other hand, a 0·2 per cent. concentration of formaldehyde in the medium gives an average of 6–7 per cent. sex-linked lethals. Further, the direct methods of formaldehyde application have a different sensitivity pattern to that obtained from formaldehyde in the larval food. Therefore, even though formaldehyde itself is probably the mutagen in both cases, differences in the mutagenic mechanisms are indicated.

Auerbach (1951) has suggested that the formaldehyde in the feeding method is reversibly bound to protein, being released either into the food, or the digestive tract, or even the germ cells themselves. However, chemical analysis of the medium has shown that only about 5 per cent. of the formaldehyde in the medium is protein-bound. This does not mean that this is not the effective mutagenic fraction but, if it is, the difference between the feeding method and direct application methods in their mutagenic effectiveness becomes even more striking. On the other hand, 40 per cent. of the formaldehyde added to the medium is not accounted for as free or protein-bound formaldehyde immediately after medium preparation. Some of this loss is due to vaporization but it is probable that most reacts with a food constituent to form a new compound. This may break down later to reform formaldehyde.

The mutation tests have shown that the rate of decrease of mutagenicity of formaldehyde media is the same whether it is preworked or not. Thus there is a decrease in mutagenic effectiveness with aging even though the chemical analysis shows there is little or no decrease in the formaldehyde concentration in the same period of time. If formaldehyde is the mutagen, one would expect the decrease in mutagenicity to be due to its loss from the medium. There is no simple reason why the formaldehyde should decrease in efficiency with no concurrent decrease in its concentration. This may mean that formaldehyde forms a reaction product in the food with something other than protein. This compound may break down with aging, releasing free formaldehyde into the food at about the same rate as it is lost by vaporization. If this is the case, then one cannot determine the real concentration of mutagenically active formaldehyde in the medium.

The results here support the conclusion of Barker and Davern (1956) that formaldehyde food does not lose its mutagenicity in 24 hr. However, Auerbach (1956) has shown that the larvae develop some sort of tolerance to the mutagenic activity of the medium in which they develop and that this tolerance develops within about 24 hr. However, this medium remains mutagenic to previously untreated larvae, while if treated larvae are transferred to fresh food, their mutation rate is further increased. It would be interesting to see if this latter occurs when treated larvae are transferred to a different vial of worked medium.

The development of this larval tolerance may help to explain the results of Herskowitz (1954). In this case, individuals 0–12 hr old (counting from time of egg laying) showed higher mutation rates than individuals 12–24 hr old. Both these groups are probably subject to the same mutagenic action for the same period of time. That is, 12–24-hr individuals will be freshly emerged larvae that will be subject to mutagenic action for 24 hr or so. Similarly, the 0–12-hr individuals will be subject to mutagen for this period when they emerge. Because of decline in the mutagenicity of the medium with time, one would expect the latter to show a lower mutation rate. The difference between these expected results and the actual ones is probably due to the method of treatment—adding an aqueous solution of formaldehyde to the top of the food in each bottle. This formaldehyde may not diffuse equally through the food in all bottles so that the results obtained could be quite fortuitous.

## V. Acknowledgments

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# EFFECTS OF X-IRRADIATION ON FOETAL DEVELOPMENT

By A. S. Fraser* and R. J. Hall*

*Summary*

Pregnant mice were irradiated at doses of 250 r from 8–11 days and 300 and 350 r from 8–14 days of pregnancy. A marked effect was found on litter size and on the number of fertilized females which produced no young at term. Three groups of facial vibrissae were scored at birth. The irradiation had a marked effect between 10–12 days of pregnancy at both 300 and 350 r, causing both a doubling and a loss of these structures.

## I. Introduction

Irradiation during early development can produce permanent modifications of the structure of a living organism. Although these effects are not heritable, they frequently copy the actions of known mutant genes, and have therefore been termed "phenocopies" (see Goldschmidt 1938).

Levine (1927), Murphy and de Renyi (1930), Job, Leibold, and Fitzmaurice (1935), Kaven (1938), Warkany and Schraffenberger (1947), and Wilson (1949) give details of the induction of phenocopies by X-irradiation, finding that these can be produced by doses as low as 100 r and that the type and frequency of abnormality is closely related to age at exposure. Russel (1950), in a large-scale series of experiments, verified these results, identifying the "sensitive periods" of a number of developmental sequences. The term "sensitive period" implied that each sequence of development passes through one or more stages during which it is sensitive to radiation.

The aims of the present study were to verify and extend the researches of previous workers, particularly those of Russel (1950), to include descriptions of the coat and skin since in her researches attention was concentrated on the internal structure of new-born mice.

## II. Material and Methods

Mice were drawn mainly from an albino crossbred stock. The date of conception was determined from the occurrence of a vaginal plug. X-rays were generated at an 85 kV peak, 5 ma, with a half-value layer of 0·6 mm aluminium. Target distance was 24·5 cm and dosage rate was 43 r per minute using an 0·5-mm aluminium filter.

The following three dosages of X-rays were used —250, 300, and 350 r. Mice, scored at birth, were classed as either alive or dead and as normal or abnormal. Gross abnormalities and presence or absence of facial vibrissae were also noted and described. Further observations were made at 3 and 6 weeks of age. In addition to verifying the earlier observations, note was taken at these ages of the structure of the coat and behaviour pattern.

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Very few abnormalities of the coat were obtained, and only three mice were noted with abnormal behaviour patterns. Attention was therefore concentrated on the presence or absence of facial vibrissae which are sensitive to radiation, easy to describe, have the advantage of occurring at several fixed positions, and are remarkably constant in number (see Dun 1958).

Three groups of facial vibrissae were scored. These are the postorbitals, supra-orbitals, and postorals, termed, for simplicity, the A, B, and C positions. Two vibrissae normally occur at each of the A and C positions, one at the B position. All three positions occur on both sides of the head, making 10 vibrissae in all.

TABLE 1

CLASSIFICATION AT BIRTH OF ALL PROGENY OF PREGNANT MICE FOR THE THREE
IRRADIATION LEVELS

Percentages given in parenthesis

| Irradiation Level (r) | Normal | | Abnormal | |
|---|---|---|---|---|
| | Alive | Dead | Alive | Dead |
| 250 | 274 (95·4) | 13 (4·6) | — | — |
| 300 | 257 (68·2) | 31 (8·2) | 42 (11·1) | 47 (12·5) |
| 350 | 222 (55·1) | 27 (6·7) | 22 (5·5) | 132 (32·7) |

## III. RESULTS AND DISCUSSION

### (a) Comparison of Dosages

The effects of the three dosages of X-rays on the progeny of the pregnant mice are given in Table 1. These results, summed over all ages at irradiation, show that the X-ray induction of phenocopies has no effect at 250 r, 24 per cent. at 300 r, and 38 per cent. at 350 r. If the mice which are apparently normal, but were born dead, are considered as "invisible" or undiagnosed phenocopies then the percentage of foetuses affected by X-irradiation are approximately 5, 32, and 45 per cent. respectively; even these can be regarded as low estimates since some of the mice born alive and classified as normal are probably undiagnosed phenocopies.

### (b) Effect of Time of Irradiation

The above data can be separated on the basis of the age at which the mice were irradiated. This is shown in Table 2. No abnormal mice were found at the 250 r dosage.

It is clear that the incidence of phenocopies increases to a maximum over 10–12 days of pregnancy. This is probably exaggerated by our preoccupation with the facial vibrissae which, as will be shown below, are maximally affected by

X-irradiation at 10–11–12 days. However, Russel (1950) found that far more characters had sensitive periods in the 10–12 day range than either earlier or later.

TABLE 2

NUMBERS OF NORMAL AND ABNORMAL MICE IN LITTERS OF PREGNANT MICE IRRADIATED AT 8–14 DAYS OF PREGNANCY

| Age at Irradiation (days) | 300 r | | | 350 r | | |
|---|---|---|---|---|---|---|
| | Normal | Abnormal | Per Cent. Abnormal | Normal | Abnormal | Per Cent. Abnormal |
| 8 | 25 | 2 | 7 | 15 | 0 | 0 |
| 9 | 17 | 2 | 11 | 15 | 4 | 21 |
| 10 | 18 | 14 | 44 | 18 | 15 | 46 |
| 11 | 85 | 27 | 24 | 0 | 25 | 100 |
| 12 | 43 | 37 | 46 | 27 | 54 | 67 |
| 13 | 79 | 7 | 8 | 87 | 41 | 32 |
| 14 | 21 | 0 | 0 | 87 | 15 | 16 |

### (c) Effect on Litter Size

The number of mice per litter, scored at birth, is shown plotted against age at irradiation for the three dosages used in Figures 1–3. A group of females from the same stock were untreated. The sizes of their litters are shown as "control" at the

TABLE 3

PERCENTAGE OF NORMAL AND ABNORMAL MICE BORN DEAD AFTER IRRADIATION OF PREGNANT MICE AT THREE LEVELS AT 8–14 DAYS OF PREGNANCY

Data from Russel (1950) shown in parenthesis

| Age at Irradiation (days) | Irradiation Level (r) | | | |
|---|---|---|---|---|
| | (200) | 250 | 300 | 350 |
| 8 | (12) | 67    (—) | 23 | 56 |
| 9 | (50) | 44    (100) | 26 | 48 |
| 10 | (61) | 66    (100) | 45 | 53 |
| 11 | (7) | 0    (92) | 28 | 100 |
| 12 | (6) | —    (40) | 36 | 82 |
| 13 | (0) | —    (—) | 14 | 36 |
| 14 | (—) | —    (—) | 6 | 8 |

right of Figure 1. The mean size of each litter is shown plotted against age at irradiation for each dosage in Figure 4.

The mean number of mice per litter from untreated females was 7·6, which is regarded as larger than usual for the white albino stock. A similar, but not identical

stock, had a mean litter size of 7·1. Even allowing the inadequacy of the control data it is clear that irradiation even at the 250 r level causes a decrease in litter size. This effect is much more marked at the 300 r level, and there is a slight increase of the effect at the 350 r level.

A noticeable feature of the data is that the litter size is more affected at the earlier than the later ages at irradiation, since many mice although they are born are already dead, and resorption of dead foetuses is more likely after early irradiation. If the differences of litter size between ages at irradiation is solely due to resorption, then we would expect the frequency of mice born dead to be proportionately greater
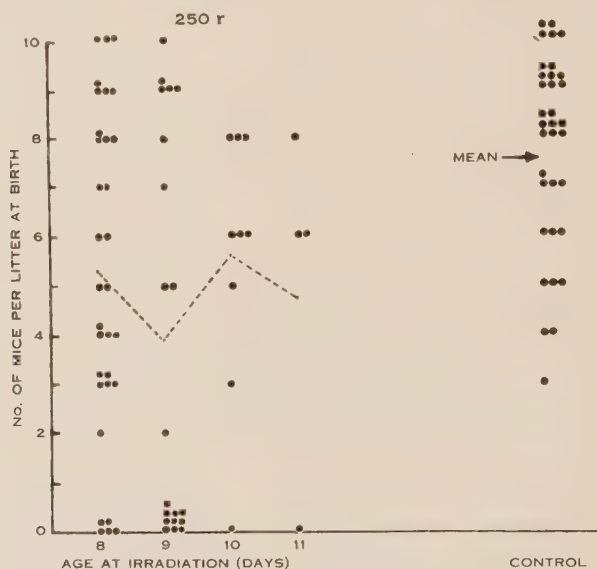


Fig. 1.—Effect of age at irradiation, in days from conception, on subsequent litter size at birth for a dosage level of 250 r. Litter sizes of unirradiated mice shown at the right of the figure. – – – – Mean litter size. ● No. of pregnant mice irradiated.

in litters of the mice irradiated at later stages of pregnancy. The percentages of mice born dead are given in Table 3.

There is no indication from these data that the percentage of dead mice is greater in litters of the mice which were irradiated at the later stages of pregnancy. It follows that the differences of litter size between different stages represent a real difference in sensitivity to radiation. The most important conclusion is that, although no abnormalities were detected at the 250 r dosage, the reduction of litter size indicates a proportion of invisible phenocopies of the order of 10–30 per cent. Further studies at lower dosages are required to examine this problem of dosage, spread over a wider range of stages of pregnancy to establish the relation of sensitivity to age at irradiation.

These results differ from those of Russel (1950) who found that "the average litter size in each of the 16 out of 18 stage-dose groups did not differ significantly from the $6 \cdot 67 \pm 0 \cdot 33$ mean of the second litter controls". Russel's data on percentage of mice born dead show a greater lethality of her treatment, which may be a sensitivity difference peculiar to her stock. Both differences can be explained by a higher rate of resorption of dead foetuses in our stock, which would give a lower litter size and a lower mortality rate.

The above analysis of effects on size of litter is based on X-irradiation affecting the individual foetus. There is, in addition, a strong indication that irradiation affects the dam causing a termination of pregnancy. Examination of the data given in Figures 1–3 shows that the number of mice which, although fertilized, produced
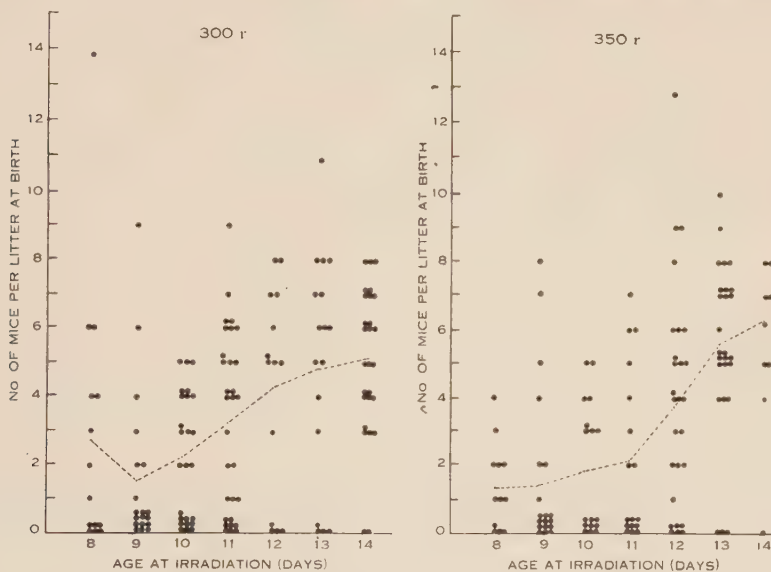


Figs. 2 and 3.—Effect of age at irradiation on subsequent litter size for dosage levels of 300 r (Fig. 2) and 350 r (Fig. 3). – – – Mean litter size. ● As for Figure 1.

no progeny at term, is greater than would be expected from a simple effect on litter size. The percentages of fertilized females which did not produce any progeny are given in Table 4.

It is clear that any effect on the dam increases with earlier age at irradiation and that it may have a maximum effect at 9 days. Russel (1950) found a marked difference in the proportion of dams which failed to produce young at term, between those irradiated between $\frac{1}{2}$–$4\frac{1}{2}$ days and those irradiated between $5\frac{1}{2}$–$8\frac{1}{2}$ days: 54 $v$. 30 per cent. In dams irradiated from $9\frac{1}{2}$–$13\frac{1}{2}$ days, 5 per cent. failed to produce young at term. It is possible that in our stock sensitivity to radiation damage of the dam is far greater, and the decrease between 9 and 8 days is only apparent. Further data are required to resolve this point.

### (d) *Effect on Facial Vibrissae*

The mean number of vibrissae at the A, B, and C positions after irradiation at the three dosage levels are given in Table 5, separated on the basis of level of dosage and age at irradiation. No effects on vibrissae followed irradiation at 250 r at any age. No effects could be detected after 13 days, i.e. number of vibrissae was normal in progeny of mice irradiated at 14 and 15 days.

Considering the totals first, it is clear that two processes are occurring. These are (i) an increase in the number of vibrissae, restricted to 9 and 10 days, and (ii) a decrease in the number of vibrissae occurring from 8–13 days.
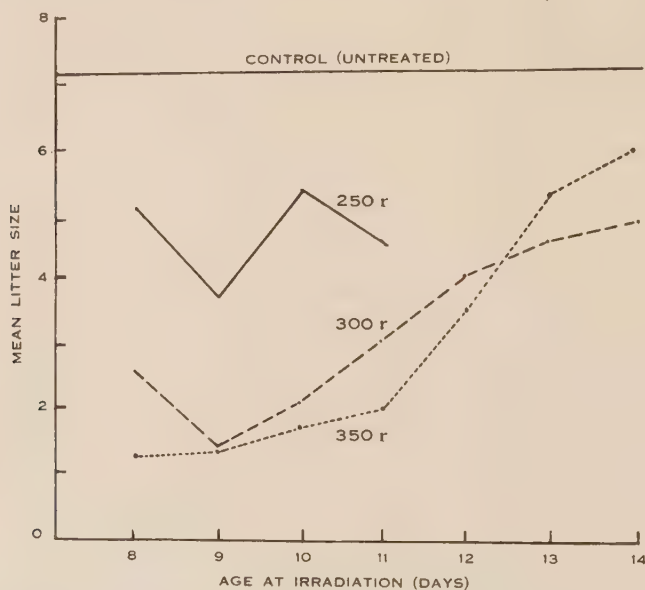


Fig. 4.— Mean litter size plotted against age at irradiation for the three radiation levels used.

The increase in number indicates that the effect of irradiation cannot be mediated solely by destruction of critical cells, unless certain cells have the function of inhibiting the doubling of follicle primordia.

The data do not show any clear relationship between the positions and the effect of irradiation at specific ages. At all three positions the sensitive period appears at the same time, 9–10 days, and the reduction phase at 11–13 days. However, reduction occurs at 8 days and it is therefore possible that reduction of vibrissae occurs at all ages from 8–13 days, but is confounded with doubling at 9–10 days. Further studies of the effect of radiation on facial vibrissae will need to attempt resolution of this problem.

An interesting feature of these effects is that reduction of the number of vibrissae occurs following irradiation at ages when no histological differentiation is visible. Development of the vibrissae primordia cannot be detected before 12 days.

As the A, B, and C positions repeat on either side of the head, it is possible to determine whether a specific sensitivity occurs by comparing the frequency of asymetric effects with symetric effects. The data are shown in Table 6, giving the

TABLE 4

NUMBERS OF FERTILIZED FEMALES WHICH DID AND DID NOT PRODUCE PROGENY AT TERM AFTER IRRADIATION AT THREE LEVELS AT 8–14 DAYS OF PREGNANCY

Percentages shown in parenthesis for "no litter" groups

| Age at Irradiation (days) | 250 r | | 300 r | | 350 r | | Mean for Three Levels of Irradiation (%) |
|---|---|---|---|---|---|---|---|
| | No. with Litter | No. with No Litter | No. with Litter | No. with No Litter | No. with Litter | No. with No Litter | |
| 8 | 27 | 5 (16) | 8 | 6 (43) | 8 | 4 (33) | 30 |
| 9 | 10 | 10 (50) | 7 | 12 (63) | 7 | 12 (63) | 59 |
| 10 | 8 | 1 (11) | 15 | 10 (40) | 8 | 9 (53) | 35 |
| 11 | 4 | 1 (20) | 22 | 8 (27) | 8 | 9 (53) | 33 |
| 12 | — | — | 10 | 4 (29) | 21 | 6 (22) | 25 |
| 13 | — | — | 13 | 4 (24) | 23 | 3 (12) | 17 |
| 14 | — | — | 25 | 2 ( 7) | 9 | 1 (10) | 9 |

frequencies of mice showing different patterns of vibrissae numbers summed over all ages, separately for the A, B, and C positions.

TABLE 5

MEAN NUMBER OF FACIAL VIBRISSAE OF PROGENY, GIVEN SEPARATELY FOR POSITIONS A, B, AND C, AND AS TOTALS OVER ALL POSITIONS, AFTER IRRADIATION OF PREGNANT MICE AT 8–14 DAYS OF PREGNANCY

| Age at Irradiation (days) | 300 r | | | | 350 r | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | Total | A | B | C | Total |
| 8 | 4·0 | 2·0 | 3·6 | 9·6 | 3·5 | 1·5 | 4·0 | 9·0 |
| 9 | 4·3 | 2·0 | 4·1 | 10·4 | 4·0 | 2·1 | 4·1 | 10·2 |
| 10 | 4·6 | 2·0 | 3·9 | 10·5 | 2·8 | 1·1 | 2·6 | 6·5 |
| 11 | 3·1 | 1·9 | 3·0 | 8·0 | — | — | — | —— |
| 12 | 3·2 | 1·8 | 3·3 | 8·3 | 2·1 | 0·7 | 1·8 | 4·6 |
| 13 | 3·6 | 1·9 | 3·4 | 8·9 | 3·5 | 1·8 | 3·5 | 8·8 |
| 14 | 4·0 | 2·0 | 4·0 | 10·0 | 4·0 | 2·0 | 4·0 | 10·0 |

These data show that there is a considerable random factor in the loss of vibrissae due to radiation. This can be seen in the large number of mice which have lost only one vibrissae. This is 21 per cent. for position A, 17 per cent. for position B,

and 34 per cent. for position C*. These proportions can be used to derive expected values for the frequency of loss of two vibrissae. These are 4, 3, and 11 per cent. respectively. The actual percentages are 27, 10, and 31. It is clear that there is a significant difference between the actual frequency of mice which have lost two vibrissae and that expected, indicating that loss of vibrissae is not solely a random process. This is further demonstrated by comparing the numbers of mice which have lost two vibrissae on one side only, with those which have lost one on each side, i.e. comparison of the 2/0 with 1/1 types. There were 22 mice with the 2/0 distribution compared with 306 with the 1/1 distribution, showing that the two vibrissae at each of the A and C positions differ in the timing of their sensitivity to radiation. This is understandable since at each of the A and C positions, one of the vibrissae starts its development 12–24 hr before the other. Russel (1950), comparing the incidence

TABLE 6

FREQUENCIES OF MICE SHOWING DIFFERENT PATTERNS OF VIBRISSAE DISTRIBUTION FOR POSITIONS A, B, AND C

No account has been taken of left v. right sides, e.g. types 2/0 and 0/2 are summed as being of type 2/0

| Position | No. of Mice with Vibrissae Distribution: | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | 3/3 | 3/2 | 2/2 | 2/1 | 2/0 | 1/1 | 1/0 | 0/0 |
| A | 2 | 4 | 575 | 123 | 7 | 150 | 24 | 8 |
| B | — | — | — | 1 | — | 608 | 106 | 63 |
| C | 3 | 1 | 417 | 142 | 15 | 156 | 37 | 24 |

of effects on the eyes, found a significant excess of animals with both eyes affected which she states "would seem to indicate a partly common sensitive area for the two eyes, or the production of a change which predisposes the embryo as a whole to eye abnormalities." The latter seems more probable.

In general, the effects of radiation on the development of vibrissae do not differ from effects on more complex structures. The "sensitive period" is of the same order of duration and the probability of damage is also in the range found for larger, multiple structures.

### (e) Effect on the Coat

Twelve newly born mice showed naked patches of skin, the affected areas showing signs of injury at birth involving formation of a scab over the area. This form of injury occurred in the progeny of mice irradiated at 350 r at 10, 11, and 12 days of pregnancy. The nakedness, unlike most genetic forms, was complete.

*Percentages calculated as follows: 2/1 as a percentage of the 2/2 type (position A), 1/0 as a percentage of the 1/1 type (position B), and 2/1 as a percentage of the 2/2 type (position C) respectively.

## (f) Behaviour Pattern

A number of newly born mice showed extreme nervousness and a tendency to throw fits. Most of these died within a week of the first manifestation of fits, at about 30–50 days after birth, all showing changes of head shape similar to the hydrocephalous genes. Two such mice survived to adult age but they were not fully grown, did not breed, and still threw fits.

## IV. Conclusions

The results obtained from these researches agree with those of previous workers (see Russel 1957), giving a picture of varying sensitivity to irradiation during pregnancy, this variation being shown as differences in pre-natal death, resorption, and the occurrence of abnormalities. Exposure to radiation at dosages in excess of 50–100 r is rare, but Russel (1957) has shown that a 25 r dose can produce quite marked effects on development. In so far as it is allowable to extrapolate from mice to humans, it is clear that the dangers of pelvic irradiation of human females during pregnancy are not negligible and require repeated emphasis (Murphy and de Renyi 1930).

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# STUDIES IN ANAPHYLAXIS

### III. RE-EXAMINATION OF SCORES FOR ANAPHYLAXIS USING FOUR INBRED LINES OF MICE

## By P. J. Claringbold* and W. R. Sobey†

#### Summary

Two significant scores were calculated from anaphylactic tests in four inbred lines of mice. The dominant score proved to be highly correlated ($r = 0.966$) with the score derived in Part I of this series (Claringbold and Sobey 1957). The implications of the second significant score are discussed.

Differences between inbred lines with respect to anaphylactic score were found and the significance of this is discussed.

Female mice were found to be more susceptible to anaphylaxis than male mice.

## I. Introduction

In Part I of this series (Claringbold and Sobey 1957), scores were calculated for different symptoms of anaphylaxis. These scores maximized the dependence of response on log-linear increment of both sensitizing and shocking doses and enabled a quantitative study of anaphylactic shock to be made. The scores were calculated on a limited amount of data, 64 random-bred mice, and may well have limited application. For any given population a score may depend on such factors as sex, genetic structure, and the type of antigen used in testing it. The present work was undertaken to examine the effect of these factors in a more detailed study.

## II. Experimental Methods and Results

### (a) Mice

Equal numbers of male and female mice from the four inbred lines $A_{Fa}$, $C57_{Fa}$, $C3H_{Fa}$, and $CBA_{Fa}$ were used in the experiment. The mice were aged from 12 to 20 weeks, housed four per box, and given standard mouse cubes and water *ad lib.*

### (b) Antigen

Bovine gamma-globulin (Armour fraction II) was used as an antigen throughout. The shocking dose, bovine gamma-globulin in 0.5 ml saline, was administered intravenously 14 days after a single, intramuscular, sensitizing injection of alum-precipitated antigen.

### (c) Experimental

The experiment was of the $4^3 \times 2$ factorial type (Cochran and Cox 1957) with two mice per treatment group (i.e. 256 mice were used in the experiment). Both sensitizing and shocking dose were given at four different levels in each of the four inbred lines and two sexes. The symptoms scored are those described in Parts I and II (Sobey and Adams 1957) and are tabulated below together with the estimated scores for the observed symptoms.

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| Designation | Scores | | | Description |
|---|---|---|---|---|
| | *1\** | *1* | *2* | |
| *0* | 0 | 0 | 0 | No effect. |
| *x* | 29 | 37 | −30 | Animal exhibits vigorous scratching of ears and face and nibbling of anal region. |
| *y* | 38 | 44 | −46 | Animal displays absence of any desire to move, huddles in a corner, with laboured breathing and infrequent convulsive movements. The animal will move when stimulated. |
| *z* | 59 | 56 | − 7 | Paralysis, partial or complete. The animal does not move when stimulated, or moves slowly, dragging its hindquarters. |
| *a* | 65 | 60 | − 1 | Death. |

Here *1\** is the score estimated in Part I, *1* is the dominant score, and *2* is the second score. The results of the experiment are shown in terms of the symptoms in Table 1, and in terms of the scores in Table 2.

## III. Statistical Analysis

The experimental data obtained are more extensive than those obtained in Part I, and manual calculation of the preliminary analysis of variance in terms of the unknowns $x$, $y$, $z$, and $a$ is very time-consuming. For the purposes of calculation, covariance analysis may be used where the observations are scored in terms of four formal variates ($x_1$–$x_4$), thus:

$$
\begin{array}{c c c c c c}
 & x_1 & x_2 & x_3 & x_4 \\
0 & 0 & 0 & 0 & 0 \\
x & 0{\cdot}5 & 0 & 0 & 0 \\
y & 0 & 0{\cdot}5 & 0 & 0 \\
z & 0 & 0 & 0{\cdot}5 & 0 \\
a & 0 & 0 & 0 & 0{\cdot}5 \\
\end{array}
$$

giving the matrices discussed in Part I. In this way it was possible to use a SILLIAC programme to carry out the calculations (Claringbold, unpublished data). The sums of squares and products of the observations were partitioned into between- and within-group components, and the between-group component was further broken down into main effects and first-order interactions, leaving higher-order interactions as a residual term.

*Matrix*

Main effects = **M**

| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | D.F. |
|---|---|---|---|---|---|
| $X_1$ | 0·657 | | | | 10 |
| $X_2$ | 0·168 | 1·374 | | | |
| $X_3$ | −0·618 | −1·011 | 2·206 | | |
| $X_4$ | −0·226 | −0·243 | 0·672 | 0·399 | |

*Within group* = **W**

$$
\begin{array}{c}
 & X_1 & X_2 & X_3 & X_4 \\
X_1 & 3{\cdot}500 \\
X_2 & -0{\cdot}875 & 4{\cdot}000 \\
X_3 & -1{\cdot}500 & -2{\cdot}375 & 6{\cdot}125 \\
X_4 & -0{\cdot}375 & -0{\cdot}250 & -1{\cdot}500 & 2{\cdot}500
\end{array}
\qquad
\begin{array}{c}
\text{D.F.} \\
128
\end{array}
$$

TABLE 1

FREQUENCY OF THE SYMPTOMS OBSERVED IN THE EXPERIMENTAL GROUPS

| Line | Sensitizing Dose (mg) | Female Shocking Dose (mg): | | | | Male Shocking Dose (mg): | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 20 | 5 | 1·25 | 0·31 | 20 | 5 | 1·25 | 0·31 |
| $A_{Fa}$ | 4 | $x+a$ | $x+z$ | $y+z$ | $x+y$ | $2y$ | $x+z$ | $x+y$ | $y$ |
| | 1 | $y+z$ | $2x$ | $2y$ | $x$ | $x+z$ | $x+a$ | $2x$ | $2x$ |
| | 0·25 | $x+z$ | $2a$ | $y+z$ | $2y$ | $2y$ | $2z$ | $x+y$ | $2x$ |
| | 0·06 | $y+z$ | $2y$ | $2y$ | $2y$ | $x+y$ | $y$ | $2y$ | $0$ |
| $C3H_{Fa}$ | 4 | $y+z$ | $z+a$ | $x+z$ | $y+z$ | $y+z$ | $2x$ | $a$ | $a$ |
| | 1 | $2y$ | $2x$ | $x+y$ | $2y$ | $y+z$ | $y+z$ | $a$ | $z+a$ |
| | 0·25 | $2y$ | $2z$ | $x+z$ | $y+z$ | $2y$ | $z$ | $z$ | $0$ |
| | 0·06 | $x+a$ | $z$ | $x+z$ | $0$ | $2y$ | $2y$ | $x$ | $0$ |
| $C57_{Fa}$ | 4 | $z+a$ | $2z$ | $y+a$ | $2y$ | $2y$ | $x+z$ | $2y$ | $x$ |
| | 1 | $2z$ | $x+z$ | $x+y$ | $2y$ | $2y$ | $x+z$ | $y+z$ | $0$ |
| | 0·25 | $2z$ | $2z$ | $y+z$ | $z$ | $y+z$ | $z$ | $2x$ | $x$ |
| | 0·06 | $x+y$ | $y$ | $x+z$ | $0$ | $2y$ | $x$ | $x+z$ | $0$ |
| $CBA_{Fa}$ | 4 | $z+a$ | $z+a$ | $z+a$ | $2z$ | $y+z$ | $2y$ | $z+a$ | $y+z$ |
| | 1 | $z+a$ | $x+a$ | $z+a$ | $2z$ | $y+z$ | $y$ | $y+z$ | $0$ |
| | 0·25 | $y+z$ | $z+a$ | $2z$ | $2z$ | $y+z$ | $z+a$ | $2y$ | $2y$ |
| | 0·06 | $y+z$ | $x$ | $z$ | $0$ | $2y$ | $2z$ | $2x$ | $2y$ |

The calculation of scores ($\mathbf{b}^T$) requires solution of the matrix equation

$$\mathbf{b}^T \left[\mathbf{M} - \theta\mathbf{T}\right] = 0,$$

where $\mathbf{T} = \mathbf{M} + \mathbf{W}$, $\mathbf{b}^T$ is a vector of estimates, $T$ indicating transposition, and $\theta$ is a root of the determinantal equation (Rao 1952)

$$\left|\mathbf{M} - \theta\mathbf{T}\right| = 0,$$

which is obtained using a SILLIAC programme.

    The roots and their test of significance are given in Table 3. The estimated scores are shown above, the first column giving those obtained in Part I. The score corresponding with the dominant root of the present investigation ($\theta_1$) is given in the second column and is clearly similar to the first estimate.

Since the variance of any score is obtained using the within-group matrix, thus:

$$\mathbf{b}^T \quad \mathbf{W} \quad \mathbf{b}/w,$$

and the covariance between a pair of scores ($\mathbf{b}$ and $\mathbf{c}$) may be obtained in the analogous fashion,

$$\mathbf{b}^T \quad \mathbf{W} \quad \mathbf{c}/w,$$

where $w$ is the within-group degrees of freedom, a correlation coefficient between

TABLE 2

EXPERIMENTAL DATA IN TERMS OF THE DOMINANT SCORE

Components of each score as in Table 1

| Line | Sensitizing Dose (mg) | Female Shocking Dose (mg) | | | | Male Shocking Dose (mg) | | | | Totals |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 20 | 5 | 1·25 | 0·31 | 20 | 5 | 1·25 | 0·31 | |
| $A_{Fa}$ | 4 | 97 | 93 | 100 | 81 | 88 | 93 | 81 | 44 | |
| | 1 | 100 | 112 | 88 | 37 | 93 | 116 | 74 | 74 | |
| | 0·25 | 93 | 120 | 100 | 88 | 88 | 112 | 81 | 74 | |
| | 0·06 | 100 | 88 | 88 | 88 | 81 | 44 | 88 | 0 | 2704 |
| $C3H_{Fa}$ | 4 | 100 | 116 | 93 | 100 | 100 | 112 | 60 | 60 | |
| | 1 | 88 | 112 | 81 | 88 | 104 | 100 | 60 | 116 | |
| | 0·25 | 88 | 112 | 93 | 100 | 88 | 56 | 56 | 0 | |
| | 0·06 | 97 | 56 | 93 | 0 | 88 | 88 | 37 | 0 | 2542 |
| $C57_{Fa}$ | 4 | 116 | 112 | 104 | 88 | 88 | 93 | 88 | 37 | |
| | 1 | 112 | 93 | 81 | 88 | 88 | 93 | 100 | 0 | |
| | 0·25 | 112 | 112 | 100 | 56 | 100 | 56 | 74 | 37 | |
| | 0·06 | 81 | 44 | 93 | 0 | 81 | 37 | 93 | 0 | 2457 |
| $CBA_{Fa}$ | 4 | 116 | 116 | 116 | 112 | 100 | 88 | 116 | 100 | |
| | 1 | 116 | 97 | 116 | 112 | 100 | 44 | 100 | 0 | |
| | 0·25 | 100 | 116 | 112 | 112 | 100 | 116 | 88 | 88 | |
| | 0·06 | 100 | 37 | 56 | 0 | 88 | 112 | 74 | 88 | 2936 |
| Totals | | 1616 | 1537 | 1514 | 1147 | 1475 | 1360 | 1270 | 718 | 10,637 |
| | | 5814 | | | | 4823 | | | | |

scores may then be calculated in the usual way. The correlation coefficient between the scores *1** and *1* is 0·966, which clearly demonstrates the equivalence of these scores.

A second latent root ($\theta_2$) is also found to be highly significant (Table 3) and the score corresponding to this root is given in the column designated 2 above. This score has zero correlation with the dominant score, a property of the method of

TABLE 3

LATENT ROOTS AND THEIR TEST OF SIGNIFICANCE .

| Latent Root | Degrees of Freedom | $\chi^2$ | $P$ |
|---|---|---|---|
| $\theta_1 = 0.5085$ | 13 | 92·5 | <0·001 |
| $\theta_2 = 0.3011$ | 11 | 46·7 | <0·001 |
| $\left.\begin{array}{l}\theta_3 = 0.1291 \\ \theta_4 = 0.0405\end{array}\right\}$ | 16 | 23·4 | 0·2–0·1 |

estimation. Information supplied by this score may be considered independently from that supplied by the dominant score. It is seen that symptoms $x$ and $y$ are the only ones scoring highly in the second variate. This indicates that irritation

TABLE 4

ANALYSIS OF VARIANCE OF THE DATA OF TABLE 1 USING THE SCORE ESTIMATED IN
PART I ($1^*$), THE DOMINANT SCORE ($1$), AND THE SECOND SCORE ($2$)

| Source of Variation | Degrees of Freedom | Mean Square | | |
|---|---|---|---|---|
| | | $1^*$ | $1$ | $2$ |
| Sex | 1 | 565*** | 380*** | 293* |
| Lines | 3 | 105** | 68* | 419* |
| Shocking dose | (3) | | | |
|    Linear | 1 | 1171*** | 1129*** | 440* |
|    Quadratic | 1 | 295** | 199** | 447* |
|    Cubic | 1 | 7 | 62 | 1557*** |
| Sensitizing dose | (3) | | | |
|    Linear | 1 | 840*** | 656*** | 58 |
|    Quadratic | 1 | 172* | 130* | 50 |
|    Cubic | 1 | 139* | 97* | 29 |
| Sex × lines | 3 | 58 | 11 | 572*** |
| Sex × shocking dose | 3 | 12 | 27 | 464** |
| Sex × sensitizing dose | 3 | 54 | 43 | 147 |
| Lines × shocking dose | 9 | 42 | 37 | 191 |
| Lines × sensitizing dose | 9 | 31 | 28 | 134 |
| Shocking × sensitizing dose | 9 | 37 | 39 | 183 |
| Residual error | 47 | 29·3 | 25·7 | 113·3 |
| Within-group error | 128 | 27·5 | 21·6 | 74·7 |

*$P < 0.05$.        **$P < 0.01$.        ***$P < 0.001$.

of the mucous membranes and the discomfort symptoms are controlled by some factors which are independent of the train of symptoms leading to death.

In Table 4 an analysis of variance is made of the data of Table 1 using the three scores. The first two columns of this table are very similar, a reflection of the high degree of correlation between the scores used. Both sexes and lines respond
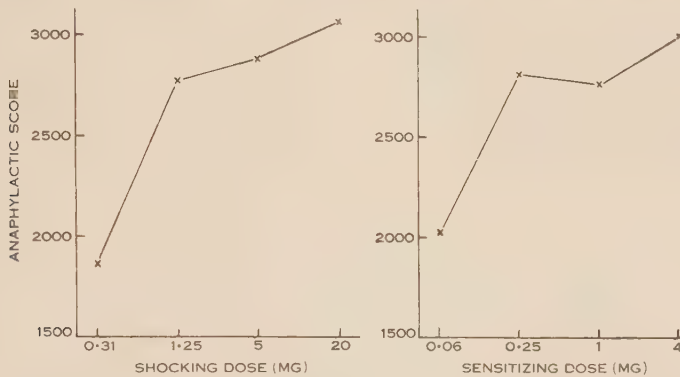


Fig. 1.—Dose response lines for shocking and sensitizing doses plotted in terms of anaphylactic score, using the dominant score.

differently to the anaphylactic reaction, females being more sensitive than males. Significant departures from linearity of response of both shocking and sensitizing dose were found. The shocking dose–response line is apparently the right-hand side

TABLE 5

ANALYSIS OF VARIANCE OF THE DATA OF PART I USING THE
SECOND SCORE (2)

| Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|
| Shocking dose | (3) | |
| Linear | 1 | 517* |
| Quadratic | 1 | 81 |
| Cubic | 1 | 14 |
| Sensitizing dose | 3 | 98 |
| Interaction | 9 | 264 |
| Error | 48 | 113 |

*$P < 0.05$.

of a sigmoid curve since the quadratic component is significant. A similar relationship of response to log-sensitizing dose was found (see Fig. 1). Since no first-order interactions were significant, these findings are consistent over lines and sexes.

Column 3 of Table 4 gives the analysis of variance of the data of Table 1 in terms of the second score. Sex and line differences are also observed with this variate, and the sex difference is not constant over lines, as evinced by the interaction

between the factors. This score has no dependence on sensitizing dose but marked dependence on shocking dose. The form of this dependence varies between the sexes.

Since only one score was found significant in Part I, the data were re-analysed using the second score (Table 5). Bearing in mind that a different antigen is being studied, a dependence (albeit linear) of response on the shocking dose is again found. Presumably the amount of information in the first experiment (64 observations) was insufficient to detect significant variation in other than the dominant score.

## IV. Discussion

The score determined in Part I and the dominant score from the present work are very highly correlated. For practical purposes, as demonstrated by comparing the first two columns in Table 4, they are interchangeable, and can be used regardless of sex or genotype. They are also valid for the two different antigens used, namely bovine plasma albumin and bovine gamma-globulin.

A second significant score, independent of the dominant score, and accentuating the values of $x$ and $y$ was found. The secondary nature of certain symptoms in anaphylaxis in dogs is reported by Dragstedt (1941). The symptoms observed were dyspnoea, vomiting, salivation, general weakness, diarrhoea, and a marked fall in blood pressure which accompanied and paralleled in degree the severity of the other symptoms. Anaesthesia prevented the vomiting, diarrhoea, etc., but did not prevent the fall in blood pressure, from which it was concluded that these reactions were probably a sequel to and less important than the vascular reaction. The second score may be of some value in physiological studies of anaphylaxis.

The shocking dose for bovine gamma-globulin is approaching a maximum at 20 mg, whereas with bovine plasma albumin a comparable value is 0·05 mg, in-dicating that different antigens may be expected to have different optimal shocking doses. Further work is being undertaken to examine this aspect of anaphylaxis.

Sobey and Adams (1957) have defined the score conferred on an animal after anaphylactic shock as the anaphylactic score. In so far as this score is dependent upon the state of the animal prior to the shocking dose, it will depend upon the extent to which the animal has been primed (by the injection of antibody or the sensitizing dose of antigen) and its innate susceptibility. This state was defined as the animal's anaphylactic potential. Different animals having the same level of available antibody may have different anaphylactic potentials because of their individual innate susceptibility to the whole anaphylactic process. This innate susceptibility was defined as the animal's anaphylactic sensitivity. If these terms are accepted, then differences in anaphylactic score between the inbred lines found here, and those reported by Fink and Rothlauf (1954) to egg albumin, indicate genetic variability in anaphylactic potential. Anaphylactic potential is probably the outcome of an interaction between the ability to produce antibodies and anaphy-lactic sensitivity. Since each of these may be under separate genetic control, line difference *per se* do not allow us to distinguish between them.

Females were found to be more susceptible to anaphylaxis than males. It is tempting to associate this with the greater antibody producing ability of females (Gorer and Schutze 1938; Sobey and Adams 1955) but the explanation is unlikely to be as simple as this since Pittman (1951) has shown females to be more sensitive than males to histamine shock, and the part played by histamine in anaphylaxis is still controversial.

## V. Acknowledgments

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# STUDIES IN ANAPHYLAXIS

## IV. THE EFFECTS OF ADJUVANTS AND OF HAEMOPHILUS PERTUSSIS ON THE ANTIGENIC PROPERTIES OF BOVINE PLASMA ALBUMIN IN THE MOUSE

By W. R. Sobey,* K. M. Adams,* and B. F. Short†

*Summary*

An optimal shocking dose of 0·05 mg for bovine plasma albumin was found over a wide range of alum-precipitated sensitizing doses.

*Haemophilus pertussis,* alum, modified Freund's adjuvant, and a mixture of all three were equally effective as adjuvants in enhancing anaphylactic shock.

Under certain conditions, *H. pertussis* was found to have a depressing effect, but otherwise a marked enhancing effect, on anaphylactic shock in actively sensitized mice.

## I. Introduction

Alum precipitation of antigen has been widely used as an adjuvant in immunological studies to enhance antibody production, and more recently to enhance anaphylactic response in the mouse (Malkiel, Hargis, and Feinberg 1953; Solotorovsky and Winsten 1953; Fink and Rothlauf 1954). There is, however, little available information on the effect of alum precipitation of the sensitizing antigen on degree of response to the shocking dose. Solotorovsky and Winsten (1953) sensitized mice with alum-precipitated antigen and then examined the effects of three shocking doses—0·25, 0·065, and 0·016 mg bovine plasma albumin. They found 0·25 and 0·065 mg gave more than 50 per cent. mortality and 0·016 mg 40 per cent. mortality. Their results do not indicate an upper inflexion point in the dose response line and it is possible that a higher shocking dose would have given a greater percentage mortality. Using the same antigen, Sobey and Adams (1957) found an optimal shocking dose of about 0·05 mg over a wide range of sensitizing doses, without alum precipitation. The apparent absence of a constant antigen-antibody ratio with respect to shocking dose could not be accounted for. It is possible that without the aid of an adjuvant the range of antibody response was so limited as not to alter the optimal shocking dose sufficiently to be noticed. If the effect of alum precipitation is markedly to increase the antibody response, and thus the amount of available antibody, then, if the optimal shocking dose is at a constant antigen-antibody ratio, the optimal shocking dose would be expected to increase.

Freund's adjuvant has been used by Wheeler, Brandon, and Petrenco (1950) to enhance anaphylactic shock to egg albumin and guinea pig serum in mice. The

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sensitizing and shocking doses used for egg albumin were large, but in spite of this, the incidence of fatal anaphylaxis was raised from 6 without, to 40 per cent. with, adjuvant. Morgan, Sherwood, and Werder (1957), using Freund's adjuvant with bovine plasma albumin, demonstrated that a high percentage mortality could be achieved. Using a shocking dose of 2 mg they obtained 93 per cent. mortality at 49 days after sensitization.

*Haemophilus pertussis*, when injected together with the sensitizing antigen, is known to enhance anaphylactic response, as originally shown by Malkiel and Hargis (1952). This enhancement is not entirely an adjuvant effect since Pittman and Germuth (1954) and Munoz, Schuchardt, and Verwey (1954) have shown that *pertussis* injected 4 days prior to shocking will enhance passive anaphylaxis.

In the present paper three sets of experiments are described. In the first the relationships of shocking and sensitizing doses, where the sensitizing antigen was alum-precipitated, are examined; in the second, the adjuvant effects of *pertussis*, modified Freund's adjuvant, and alum are compared; and in the third, further experiments on the effect of *pertussis* in passive and active anaphylaxis are carried out.

## II. Materials and Methods

### (a) Mice

Female mice weighing about 25 g, from a randomly bred albino stock maintained in this laboratory, were used throughout. The mice were given standard mouse cubes and water *ad lib.*

### (b) Antigen

Bovine plasma albumin (B.P.A.) (Armour fraction V) was used throughout. All shocking doses were administered by the intravenous injection of 0·5 ml of the appropriate amount of antigen made up in 0·9 per cent. saline.

The method of Proom (1943) was followed with respect to alum precipitation. Alum (potassium aluminium sulphate) (2·5 g) was dissolved in 100 ml 1 per cent. B.P.A. and precipitation effected by the slow addition, with stirring, of 1N NaOH to pH 6·8. The precipitate was centrifuged, washed once in 0·9 per cent. saline, and resuspended in 20 ml saline. This suspension was then assumed to contain 50 mg B.P.A. per ml.

Freund's adjuvant could not be obtained commercially and was made up in the following modified form: Lanoline was added to low-viscosity oil (Shell "Risella 17") in the ratio 1 : 4. Dried, killed mycobacteria (a mixture of *phlei* and *butyricum*) was added to give a final concentration of 2 mg/ml. Antigen of the appropriate concentration in 0·9 per cent. saline was added to this in equal volumes and the mixture emulsified in a blendor. The emulsion was found to be stable over a period of weeks.

Phase I *H. pertussis* was obtained from Commonwealth Serum Laboratories, Parkville, Vic., in suspensions at a concentration of $1 \times 10^{12}$ organisms/ml.

### (c) Preparation of Antiserum

Sixty mice were given a single injection of 5 mg alum-precipitated B.P.A. intramuscularly and bled 14 days later by the method described by Sobey and

TABLE 1

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 1

Each treatment group is the sum of four observations

| Sensitizing Dose (mg) | Times of Testing | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | 14 Days | | | | | 28 Days | | | | |
| | Shocking Dose (mg) | | | | | Shocking Dose (mg) | | | | |
| | 0·1 | 0·5 | 2·5 | 12·5 | Total | 0·1 | 0·5 | 2·5 | 12·5 | Total |
| 0·25 | 254 | 168 | 176 | 148 | 746 | 248 | 220 | 148 | 111 | 727 |
| 1·0 | 226 | 232 | 198 | 148 | 804 | 260 | 254 | 170 | 148 | 832 |
| 4·0 | 254 | 226 | 198 | 148 | 826 | 254 | 242 | 226 | 192 | 914 |
| 16·0 | 254 | 260 | 198 | 170 | 882 | 260 | 226 | 260 | 198 | 944 |
| Totals | 988 | 886 | 770 | 614 | 3258 | 1022 | 942 | 804 | 649 | 3417 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Mean Square | F |
| --- | --- | --- | --- |
| Shocking dose (A) | (3) | | |
|    Linear | 1 | 9727 | 98·1*** |
|    Remainder | 2 | 65 | 1 |
| Sensitizing dose (B) | (3) | | |
|    Linear | 1 | 2113 | 21·3*** |
|    Remainder | 2 | 25 | 1 |
| Times of testing (C) | 1 | 198 | 2 |
| First-order interactions | | | |
|    A × B | 9 | 100 | 1 |
|    A × C | 3 | 4 | 1 |
|    B × C | 3 | 67 | 1 |
| Second-order interactions | | | |
|    A × B × C | 9 | 178 | 1·8 |
| Error | 96 | 99 | |

*** $P < 0.001$.

Adams (1955). Blood was collected in heparinized tubes for convenience of handling. After centrifugation the clear plasma was stored at $-10°C$.

### (d) Quantitative Score

The score as described in Part III of this series (Claringbold and Sobey 1958, p. 435) was used throughout.

### (e) Experimental Design

All the experiments described are of factorial design, and since the treatment variables are not constant they are detailed for each experiment in the text.

## III. RESULTS

### (a) Sensitizing and Shocking Dose Responses where the Sensitizing Antigen was Alum-precipitated

(i) *Experiment 1.*—Sensitizing and shocking doses were each varied at four levels. The sensitizing dose, given in a single intramuscular injection, was so
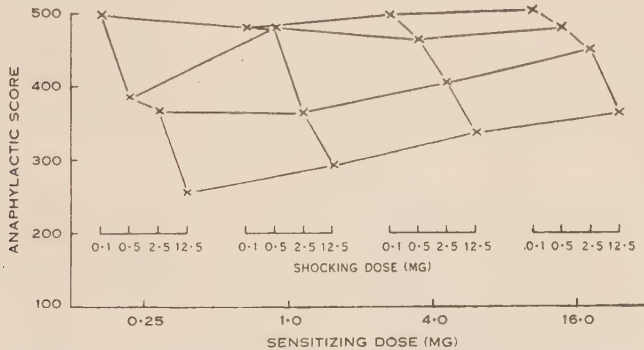


Fig. 1.—Sensitizing and shocking dose responses of experiment 1 plotted as a surface.

scheduled that half the animals were sensitized 14 days, and half 28 days, prior to shocking. This gave a $4^2 \times 2$ factorial design, and with four animals per treatment group 128 animals in all were used. The design, results, and analysis of variance are given in Table 1.

Anaphylactic score decreased log-linearly with increased shocking dose, and increased log-linearly with increased sensitizing dose. The dose responses for both shocking and sensitizing doses are illustrated as a surface in Figure 1, where the data have been summed over the two times of testing (14 and 28 days) since these were not significantly different. There were no significant interactions.

In view of the absence of an inflexion point on the shocking dose response line a further experiment was undertaken with a wider range of shocking dose.

(ii) *Experiment 2.*—Sensitizing and shocking doses were each varied at five levels, and there were five animals per treatment group. This gave a $5^2$ factorial design with 125 mice in the experiment. The design, results in full, and analysis of variance are given in Table 2.

Anaphylactic score increased log-linearly with increased sensitizing dose. The shocking dose response line is markedly quadratic with the inflexion point at

| Sensitizing Dose (mg) | Shocking Dose (mg) | | | | | Totals |
|---|---|---|---|---|---|---|
| | 0·002 | 0·01 | 0·05 | 0·25 | ·1·25 | |
| 0·08 | 38 | 38 | 38 | 38 | 38 | |
| | 38 | 38 | 59 | 38 | 0 | |
| | 38 | 59 | 65 | 38 | 38 | |
| | 0 | 59 | 65 | 38 | 38 | |
| | 65 | 38 | 65 | 38 | 38 | |
| Totals        ..        .. | 179 | 232 | 292 | 190 | 152 | 1045 |
| 0·31 | 0 | 65 | 65 | 38 | 38 | |
| | 29 | 38 | 38 | 65 | 38 | |
| | 29 | 29 | 65 | 65 | 38 | |
| | 65 | 65 | 59 | 38 | 59 | |
| | 38 | 65 | 65 | 38 | 65 | |
| Totals        ..        .. | 161 | 262 | 292 | 244 | 238 | 1197 |
| 1·25 | 38 | 59 | 38 | 65 | 38 | |
| | 29 | 38 | 65 | 59 | 38 | |
| | 38 | 59 | 65 | 59 | 38 | |
| | 59 | 65 | 65 | 65 | 38 | |
| | 38 | 65 | 65 | 65 | 65 | |
| Totals        ..        .. | 202 | 286 | 298 | 313 | 217 | 1316 |
| 5·0 | 38 | 65 | 65 | 65 | 38 | |
| | 38 | 65 | 65 | 65 | 38 | |
| | 38 | 65 | 59 | 65 | 38 | |
| | 59 | 65 | 65 | 38 | 38 | |
| | 29 | 38 | 65 | 65 | 38 | |
| Totals        ..        .. | 202 | 298 | 319 | 298 | 190 | 1307 |
| 20·0 | 29 | 38 | 65 | 65 | 38 | |
| | 38 | 65 | 65 | 65 | 65 | |
| | 38 | 59 | 65 | 65 | 65 | |
| | 29 | 59 | 65 | 38 | 65 | |
| | 29 | 59 | 65 | 65 | 65 | |
| Totals        ..        .. | 163 | 280 | 325 | 298 | 298 | 1364 |
| Grand totals        .. | 907 | 1358 | 1526 | 1343 | 1095 | 6229 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Mean Square | $F$ |
|---|---|---|---|
| Sensitizing dose (A) | (4) | | |
| Linear | 1 | 224 | 14·45*** |
| Remainder | 3 | 13 | <1 |
| Shocking dose (B) | (4) | | |
| Quadratic | 1 | 874 | 56·41*** |
| Remainder | 3 | 26 | 1·73 |
| Interactions   A × B | 16 | 18 | 1·18 |
| Error | 100 | 15 | |

*** $P < 0.001$.

0·05 mg. There were no significant interactions. Sensitizing and shocking dose responses are illustrated as a surface in Figure 2. The data are given in full in Table 2 to indicate clearly the high degree of mortality at the higher sensitizing
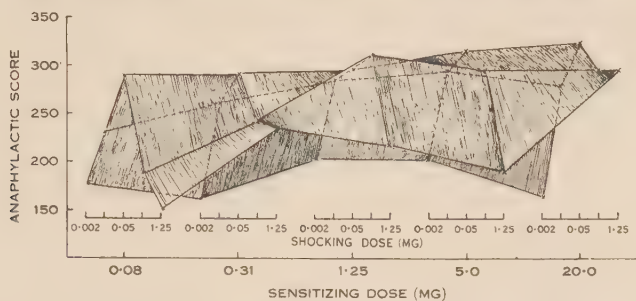


Fig. 2.—Sensitizing and shocking dose responses of experiment 2 plotted as a surface.

doses and to complement Figure 2, in illustrating that while the shocking dose of 0·05 mg is almost uniformly optimal, it is not significantly different from 0·25 and 1·25 mg at the highest sensitizing dose.
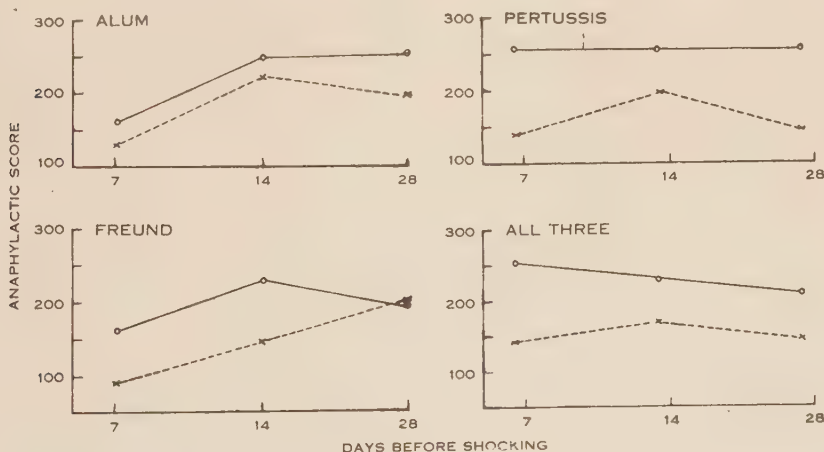


Fig. 3.—Individual dose response lines of the four treatments of experiment 3. Shocking doses: ○ 0·5 mg; × 5 mg.

(b) *A Comparison of the Adjuvant Effects of* pertussis, *Modified Freund's Adjuvant, and Alum-precipitated Antigen*

(i) *Experiment 3.*—In this experiment alum, modified Freund's adjuvant, *pertussis*, and a mixture of all three were used as adjuvants. A single 0·1 ml sensitizing injection of each, containing 5 mg B.P.A. made up in the appropriate adjuvant, was administered intramuscularly. Where *pertussis* alone was used the dose consisted of 0·1 ml of a suspension containing $1 \times 10^{12}$ organisms/ml. The mixture of all three adjuvants thus contained one-third of this amount *pertussis* since it was

TABLE 3

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 3

Each treatment combination is the sum of four observations

| Times of Sensitizing prior to Shocking (days) | Shocking Dose (mg) | Adjuvants | | | | Totals |
|---|---|---|---|---|---|---|
| | | Alum | Freund | *pertussis* | All Three | |
| 7 | 5 | 134 | 96 | 141 | 143 | 514 |
| | 0·05 | 161 | 162 | 260 | 254 | 837 |
| | | 295 | 258 | 401 | 397 | 1351 |
| 14 | 5 | 227 | 152 | 200 | 173 | 752 |
| | 0·05 | 254 | 233 | 260 | 233 | 980 |
| | | 481 | 385 | 460 | 406 | 1732 |
| 28 | 5 | 200 | 206 | 152 | 152 | 710 |
| | 0·05 | 260 | 200 | 260 | 206 | 926 |
| | | 460 | 406 | 412 | 358 | 1636 |
| Totals | | 1236 | 1049 | 1273 | 1161 | 4719 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Mean Square | F |
|---|---|---|---|
| Adjuvants (A) | 3 | 4,070 | 2·5 |
| Shocking doses (B) | 1 | 61,281 | 37·5*** |
| Times of shocking (C) | (2) | | |
|    Linear | 1 | 12,692 | 7·8** |
|    Quadratic | 1 | 11,850 | 7·2** |
| First-order interactions | | | |
|    A × B | 3 | 2,611 | 1·6 |
|    A × B | 6 | 3,587 | 2·2 |
|    B × C | 2 | 1,074 | <1 |
| Second-order interactions | | | |
|    A × B × C | 6 | 1,513 | <1 |
| Error | 72 | 1,636 | |

** $P < 0·01$.      *** $P < 0·001$.

made up from an equal volume of each adjuvant. The sensitizing schedule was so arranged that one-third of the mice were injected 7 days, one-third 14 days, and the remaining third 28 days prior to shocking. Two shocking doses, 5 and 0·05 mg,

were used. The experiment was thus a $4 \times 3 \times 2$ factorial design and with four mice per treatment group made up a total of 96 mice. The design, results, and analysis of variance are given in Table 3.

There was no significant difference between the four treatments. The scores for the two shocking doses were significantly different, $0 \cdot 05$ mg giving a higher score than 5 mg (see Table 3). Times of sensitizing prior to shocking were significantly different. Response rises to a peak at 14 days and then flattens out. The individual dose-time-response lines for the adjuvant treatments are illustrated in Figure 3. At 7, 14, and 28 days those mice sensitized with *pertussis* as an adjuvant and shocked with $0 \cdot 05$ mg were all fatally shocked.
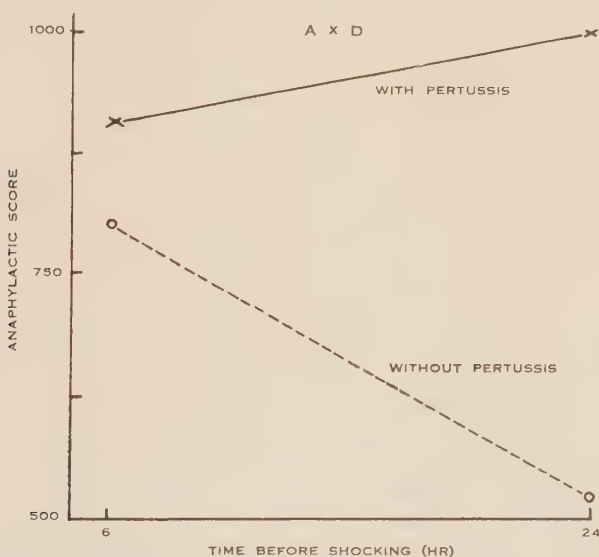


Fig. 4.—Interactions of time between sensitizing and shocking dose and treatment with *pertussis* ($A \times D$) from experiment 4.

### (c) *The Effects of* pertussis *under Varied Conditions*

(i) *Experiment* 4.—The effect of *pertussis* in passive anaphylaxis was the main consideration of this experiment. It was of a $2^4$ factorial design with four animals per treatment group, i.e. 64 mice in all. The shocking doses were $0 \cdot 5$ and $0 \cdot 05$ mg. Sensitizing doses of $0 \cdot 5$ and 1 ml of mouse anti-B.P.A. were given to half the mice 6 hr, and half 24 hr, prior to shocking. Half the mice were given an intramuscular injection of $0 \cdot 1$ ml *pertussis* suspension containing $1 \times 10^{12}$ organisms/ml, 4 days prior to shocking. The design, results, and analysis of variance are given in Table 4.

The average score at 6 hr was significantly higher than at 24 hr. Neither the shocking nor sensitizing doses were significantly different in their effect. *pertussis*-treated animals gave a significantly higher score than untreated animals. One first-order interaction, that between *pertussis* and time of shocking ($A \times D$), was significant. This, illustrated in Figure 4, is the result of a marked difference

TABLE 4

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 4

Each treatment group is the sum of four observations

| Times of Shocking after Sensitizing | Shocking Dose (mg) | Sensitizing Dose (ml) | With *pertussis* | Without *pertussis* | Totals |
|---|---|---|---|---|---|
| 6 hr | 0·5 | 0·5 | 206 | 221 | 427 |
|  |  | 1 | 212 | 179 | 391 |
| 6 hr | 0·05 | 0·5 | 260 | 215 | 475 |
|  |  | 1 | 254 | 194 | 448 |
| Totals |  |  | 932 | 809 | 1741 |
| 24 hr | 0·5 | 0·5 | 233 | 114 | 347 |
|  |  | 1 | 260 | 152 | 412 |
| 24 hr | 0·05 | 0·5 | 260 | 143 | 405 |
|  |  | 1 | 260 | 114 | 374 |
| Totals |  |  | 1013 | 523 | 1536 |
| Grand totals |  |  | 1945 | 1332 | 3277 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Mean Square | $F$ |
|---|---|---|---|
| Times of shocking (A) | 1 | 863 | 7·8** |
| Shocking dose (B) | 1 | 135 | 1·2 |
| Sensitizing dose (C) | 1 | 0 | 1 |
| *pertussis* treatment (D) | 1 | 6460 | 58·6*** |
| First-order interactions |  |  |  |
| A × D | 1 | 1775 | 16·1*** |
| Remainder | 5 | 108 | 1 |
| Second-order interactions | 4 | 47 | 1 |
| Third-order interactions | 1 | 162 | 1·6 |
| Error | 48 | 110 |  |

** $P < 0.01$.          *** $P < 0.001$.

in the slopes of the time response lines. *pertussis*-treated animals gave a higher score at 24 hr than at 6 hr whereas with untreated animals it was the reverse. In the absence of *pertussis*, anaphylactic potential falls off with time from 6 hr after sensitization whereas in the presence of *pertussis* there is an increase in potential with time up to, and possibly beyond, 24 hr. The reason for this is obscure. It

may be due to some interaction of *pertussis* and antibody which requires time to reach its maximal effect.

(ii) *Experiment 5.*—This experiment was a $2^5$ factorial design with four mice per treatment group, making up 128 mice. Sensitizing doses of 5 and 0·05 mg and shocking doses of 0·5 and 0·05 mg were used. Half the mice received their last sensitizing injection 7 days, and half 14 days, prior to shocking. The sensitizing dose was given either in a single injection or in two injections spaced by 1 week. All sensitizing injections were given intramuscularly. One week prior to shocking half the mice were injected intraperitoneally with 0·1 ml of a *pertussis* solution containing $18 \times 10^9$ organisms/ml. The design, results, and analysis of variance are given in Table 5.
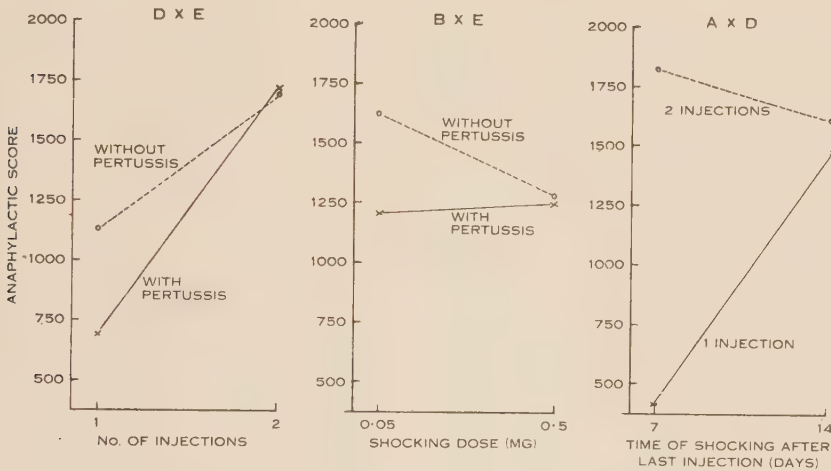


Fig. 5.—Interactions of treatment with *pertussis* and number and level of shocking dose (D × E, B × E) and of number of injections and time between shocking and sensitizing dose (A × D) from experiment 5.

The score at 14 days is significantly higher than at 7 days. Differences between shocking doses, significant at the 5 per cent. level, indicate 0·05 mg to have a higher score than 0·5 mg. While the two levels of sensitizing injection were not significantly different, two injections gave a significantly higher score than a single injection where the same total sensitizing dose was used. Animals given *pertussis* (in this instance given separately from the antigen by the intraperitoneal route) and in lower concentration than in experiment 3, had lower scores than untreated animals. There were two first-order interactions involving *pertussis*: the presence or absence of *pertussis* with the number of injections (D × E), and with shocking dose (B × E). These are illustrated in Figure 5. The first shows that *pertussis* only had a depressing effect on anaphylactic score where a single sensitizing injection was given. This suggests that the depressing effect is associated with the level of antibody response and is only apparent where the response is low. The interaction of *pertussis* with shocking dose indicates that the concentration of the shocking dose is less important in *pertussis*-treated than in untreated animals. The third significant first-order

TABLE 5

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 5

Each treatment combination is the sum of four observations

| *pertussis* | No. of Injections | Sensitizing Dose (mg) | Shocking Dose 7 Days after Sensitizing (mg) | | Shocking Dose 14 Days after Sensitizing (mg) | | Totals |
|---|---|---|---|---|---|---|---|
| | | | 0·5 | 0·05 | 0·5 | 0·05 | |
| With | 1 | 5 | 0 | 29 | 170 | 168 | |
| | | 0·05 | 29 | 29 | 132 | 148 | |
| | Total | ..    .. | 29 | 58 | 302 | 316 | 705 |
| | 2 | 5 | 260 | 214 | 220 | 162 | |
| | | 0·05 | 204 | 260 | 236 | 192 | |
| | Total | ..    .. | 464 | 474 | 456 | 354 | 1748 |
| Totals | | | 493 | 532 | 758 | 670 | 2453 |
| Without | 1 | 5 | 74 | 95 | 204 | 254 | |
| | | 0·05 | 58 | 58 | 176 | 248 | |
| | Total | ..    .. | 132 | 153 | 380 | 502 | 1167 |
| | 2 | 5 | 220 | 254 | 224 | 254 | |
| | | 0·05 | 198 | 242 | 132 | 220 | |
| | Total | ..    .. | 418 | 496 | 356 | 474 | 1744 |
| Totals | | | 550 | 649 | 736 | 976 | 2911 |
| Grand totals | | | 1043 | 1181 | 1497 | 1646 | 5364 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Mean Square | F |
|---|---|---|---|
| Time of testing (A) | 1 | 6,555 | 46·3*** |
| Shocking dose (B) | 1 | 657 | 4·6* |
| Sensitizing dose (C) | 1 | 450 | 3·2 |
| No. of injections (D) | 1 | 220,504 | 144·7*** |
| *pertussis* (E) | 1 | 1,637 | 11·5** |
| First-order interactions | | | |
|     A × B | 1 | 2 | <1 |
|     A × C | 1 | 85 | <1 |
|     A × D | 1 | 14,028 | 99·0*** |
|     A × E | 1 | 95 | <1 |
|     B × C | 1 | 237 | <1·7 |
|     B × D | 1 | 53 | <1 |
|     B × E | 1 | 1,176 | 8·3** |
|     C × D | 1 | 0 | <1 |
|     C × E | 1 | 504 | 3·6 |
|     D × E | 1 | 1,697 | 12·0*** |
| Second-order interactions | 10 | 154 | 1·1 |
| Error | 96 | 142 | |

    * $P < 0.05$.       ** $P < 0.01$.       *** $P < 0.001$.

interaction was between the number of sensitizing injections and the time of shocking after the last injection (A × D). This suggests that the maximum score can be expected 14 days after the first injection where two injections are given, the second injection being responsible for rise in score at this point but having no marked effect on the time of optimal response.

(iii) *Experiment* 6.—To obtain further information on the effect of *pertussis* the following experiment was undertaken. It was a $2^2 \times 3$ factorial design with five animals per treatment group, i.e. 60 mice in all were used. The primary aim was to obtain information on the effects of *pertussis* other than those of an adjuvant.
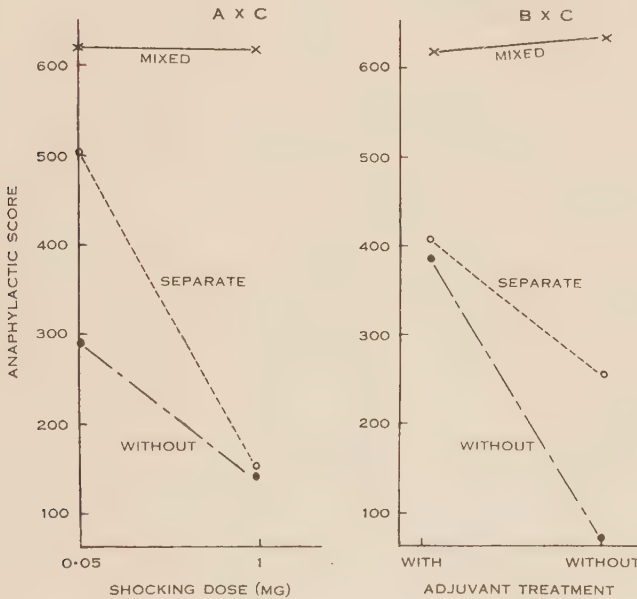


Fig. 6.—Interactions of treatment with *pertussis* and shocking dose (A × C) and of treatment with *pertussis* and adjuvant treatment (B × C) from experiment 6.

Two levels of shocking dose, 1 and 0·05 mg, and two adjuvant treatments, with and without alum precipitation, were used. Three *pertussis* treatments were used: (1) the antigen was mixed with *pertussis* prior to injection; (2) *pertussis* and antigen were injected at different sites (opposite hind legs); (3) no *pertussis* was given. All sensitizing injections were of 5 mg and were administered intramuscularly. For all *pertussis* treatments, 0·1 ml of a suspension containing $1 \times 10^{12}$ organisms/ml was used. Animals were subjected to shocking doses 14 days after sensitization. The design, results, and analysis of variance are given in Table 6.

The shocking doses were significantly different, 0·05 giving a higher score than 1 mg. Alum-precipitated antigen resulted in a significantly higher score than untreated antigen. The three *pertussis* treatments were significantly different, the highest score being achieved where *pertussis* was mixed; here almost uniform fatality

resulted. *pertussis* given in separate sites gave a higher score than no treatment. The reason for this apparent contradiction of the previous experiment is not clear; it may be associated with the use of a different route of injection, concentration of *pertussis*, and time of administering *pertussis*.

TABLE 6

DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF EXPERIMENT 6

Each treatment combination is the sum of five observations

| Shocking Dose (mg) | Adjuvant Treatment | *pertussis* Treatment | | | Totals |
| --- | --- | --- | --- | --- | --- |
| | | Mixed | Separate | Without | |
| 1 | With alum pptn. | 319 | 94 | 125 | 538 |
| | Without alum pptn. | 319 | 65 | 29 | 413 |
| | | 638 | 159 | 154 | 951 |
| 0·05 | With alum pptn. | 315 | 319 | 269 | 903 |
| | Without alum pptn. | 325 | 195 | 29 | 549 |
| | | 640 | 514 | 298 | 1452 |
| Totals | | 1278 | 673 | 452 | 2403 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Mean Square | F |
| --- | --- | --- | --- |
| Shocking dose (A) | 1 | 4150 | 11·9** |
| Adjuvant (B) | 1 | 3792 | 10·9** |
| *pertussis* (C) | 2 | 9095 | 26·1*** |
| First-order interactions | | | |
| A × B | 1 | 859 | 2·5 |
| A × C | 2 | 1594 | 4·6* |
| B × C | 2 | 1515 | 4·4* |
| Second-order interactions | | | |
| A × B × C | 2 | 318 | <1 |
| Error | 48 | 348 | |

\* $P < 0·05$.      \*\* $P < 0·01$.      \*\*\* $P < 0·001$.

Two first-order interactions involving *pertussis* were significant at the 5 per cent. level, namely *pertussis* × shocking dose (A × C) and *pertussis* × adjuvant treatment (B × C), and these are illustrated in Figure 6. In both instances the interaction appears to be the result of marked differences in the slope of the dose response lines. When mixed with the antigen the *pertussis*-treated mice were all fatally

shocked irrespective of the shocking dose, or whether the antigen was alum-precipitated or not. Animals which were treated with *pertussis* in a separate site from the antigen gave higher scores than untreated animals and were sensitive to the optimal shocking dose (A×C, Fig. 6); they gave a higher score than the untreated animals, where no alum was used.

## IV. DISCUSSION

It is clear from our data and that of Solotorovsky and Winsten (1953), Malkiel, Hargis, and Feinberg (1953), Morgan, Sherwood, and Werden (1957), and others that adjuvants, in general, greatly enhance anaphylactic shock. Since adjuvants are known to enhance antibody response it is reasonable to argue that increased anaphylactic potential (Sobey and Adams 1957) is a direct consequence. An optimal shocking dose of 0·05 mg was found over a wide range of alum-precipitated sensitizing doses. The same optimal shocking dose was found by Sobey and Adams (1957) over a wide range of sensitizing doses in the absence of an adjuvant. This strongly suggests that the size of the shocking dose is the important factor and not an optimal antigen-antibody ratio, as might be expected. The reasons for this are at present inexplicable.

Alum precipitation, modified Freund's adjuvant, *pertussis*, and a mixture of all three of these proved to have similar capacities for enhancing anaphylactic shock under the conditions of testing. Morgan, Sherwood, and Werder (1957), using Freund's adjuvant, obtained their highest percentage mortality 35–42 days post-sensitization using a shocking dose of 2 mg B.P.A. In view of this evidence it is probable that the modified Freund's adjuvant would have proved more effective in our tests had they been made over a greater period of time after sensitization. Such long time intervals are often inconvenient. If it is merely desired to obtain a high percentage mortality this can readily be achieved in 7 days using *pertussis*, or 14 days using Freund's adjuvant or alum, provided the optimal shocking dose is used.

When *pertussis* was injected 4 days before shocking in passive anaphylaxis (expt. 4) it markedly increased anaphylactic potential. A similar increase in potential was observed in active anaphylaxis where *pertussis* and antigen were injected at different sites 14 days prior to shocking. These observations suggest that *pertussis* may enhance anaphylactic potential by more than just the enhancing of antibody response. Malkiel (1956) has shown that the effect of *pertussis* is not the result of a direct toxic effect on the adrenal gland. In view of the evidence of Riley (1953) on the histamine content of mast cells, and that of Freund and Stone (1956) on the production of Arthus reaction in the lips of mice and rats, areas rich in mast cells, it is tempting to speculate that the action of *pertussis* may in some way be associated with the sensitivity or multiplication of cells responsible for the production or storage of agents concerned in the shock reaction (histamine, serotonin, etc.). The observation in experiment 5 that *pertussis* does not always enhance, and may even depress, anaphylactic potential emphasizes our lack of knowledge of its mode of action and the need for detailed observations on the effects of dose, route of injection, and time of injection prior to shocking.

## V. Acknowledgments

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# AN OSMOTIC MUTANT OF *ARABIDOPSIS THALIANA*

## By J. Langridge*

*Summary*

A single recessive mutation in *Arabidopsis thaliana* (L.) Heynh. causes, at 23°C, distorted growth, narrower leaves, poor secondary root development, and complete sterility. Mutant plants grown at 18°C are very small with little differentiation and soon die, but at 28°C development is normal. The aberrant phenotype of the mutant is a consequence of its abnormally low cellular osmotic pressure, for, in aseptic culture at 23°C, additions to the medium of glucose, sucrose, or potassium sulphate, in amounts giving a substrate osmotic pressure greater than 2·5 atm, restore normal growth. Phenocopies induced by depressing the effective osmotic pressure of wild-type plants by growing them on mannitol closely resemble mutant plants in most respects. Glucose and sucrose both increase leaf width, but while the former substance increases only cell division, the latter's action is primarily to increase cell expansion. An incompatibility system is a secondary consequence of the mutation, for mutant pollen fails to grow on mutant stigmas, but will grow on wild-type stigmas; the reverse pollination is ineffective. Fertility in the mutant is restored by raising the substrate osmotic pressure, the relationship between osmotic pressure of medium as given by glucose and the number of seeds set per plant being linear. No differences are apparent in the carbohydrate metabolism or cell permeability of the mutant, and no cause could be found for its low osmotic pressure.

## I. INTRODUCTION

One of the major aims in physiological genetics is the tracing of the reaction chains which link the presence of a gene with a morphological phenotype. The general method of approach is ideally that of working back through decreasing levels of phenotypic complexity—morphological, anatomical, physiological, and eventually to the biochemical steps governed by single genes. This approach has been valuable in showing how genic interference in a basic reaction of development may, by upsetting the balance between subsequent morphogenic processes, give rise to very complex phenotypes. In practice, however, the analysis often stops at some level in the development of the phenotype that may be far removed from the primary developmental action of the gene. Usually, it is not possible to find the physiological difference that causes an anatomical change, probably because of the lack of knowledge of specific chemical activators in differentiation. This situation is particularly evident in the flowering plants, where mutant phenotypes seldom receive more than a general morphological or, at best, a histological description. The studies reported in this paper represent an attempt to provide a more detailed explanation of the action of a gene in controlling morphogenesis.

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## II. Material and Methods

The mutant under study (designated 1023/13) was first isolated from $X_2$ plants of *Arabidopsis thaliana* (L.) Heynh. growing on minimal agar medium containing 2 per cent. sucrose. It appeared as a plant smaller than the wild type, slower in flowering, and with pointed leaves. When grown on minimal medium lacking sucrose, mutant plants produced small, narrow, twisted, asymmetrical leaves, multiple short flower stalks, and failed to form seed. In the $X_2$ generation the ratio of wild type to mutant was 43 : 6, closely conforming to the expected 7 : 1 ratio for a mutation arising in one cell of a two-celled apical meristem in the irradiated seed. The $F_2$ segregation ratio of 57 : 14 ($\chi^2$ for 3 : 1 = 1·06, $P = 0·4–0·3$) indicates that the mutant phenotype results from a single gene change. No back mutations to wild type appeared in over 700 plants studied.

Mutant plants do not survive to flowering when grown in soil. Initial growth in soil is usually quite good, plants having normal-shaped leaves one-half to two-thirds the size of the wild type. However, after the first two or three pairs of leaves, differentiation becomes abnormal and plants soon die.

The mutant was examined on plants grown aseptically on chemically defined media under conditions described by Langridge (1957, 1958). Most growth experiments were made with substrates with the tonicity altered by potassium sulphate glucose, sucrose, or mannitol. The glucose and potassium sulphate used were of "analytical reagent" grade, while sucrose solutions were purified by boiling with activated charcoal for 5 min. Commercial mannitol was quite toxic to the plants unless it was first purified by extraction with ether at pH 3 and then at pH 8 and recrystallized.

## III. Results

During the process of isolation and initial testing it was noted that, although the mutant showed extreme disorganization of growth when grown on minimal medium, its phenotype when grown on sucrose-containing substrate was almost that of the wild type. Growth was not improved by supplements such as coconut milk, amino acids, vitamins, yeast extract, and nucleic acid hydrolysate.

### (a) The Effect of Altering the Osmotic Pressure of the Substrate

As the preliminary tests indicated that the mutation had induced a requirement for sucrose, experiments were conducted to distinguish between the nutritional and osmotic effects of the sugar. For this purpose, mutant plants were grown on minimal agar plus sucrose, glucose, potassium sulphate, or mannitol, each added in amounts calculated to give substrates with an osmotic pressure of 2 atm. The osmotic pressure of the minimal medium was approximately 0·5 atm.

(i) *Penetrance and Expressivity of the Mutant Genotype.*—As may be seen from Plate 1, which illustrates mutant plants (26 days old) on different concentrations of sucrose, there may be a complete gradation in phenotype from a very small lethal form, to one which closely approaches that of the wild type. There is also variation in phenotypic expression (expressivity) with any treatment which, in general, tends to favour the growth of the mutant. The number of homozygous

recessive individuals which exhibit an abnormal phenotype with a given treatment represents the penetrance of the mutant gene under these conditions. As may be seen from Table 1, the expressivity of the gene varies with the treatment and increases as growth proceeds, while Figures 1 and 2 show that its penetrance decreases as the osmotic pressure of the medium increases up to 2·5 atm.

TABLE 1

EFFECT OF OSMOTIC AGENTS ON THE EXPRESSION OF THE MUTANT PHENOTYPE

Temperature 23°C

| Osmotic Agent | Osmotic Pressure of Substrate (atm) | No. of Plants | Age of Plants (days) | Phenotype | | |
|---|---|---|---|---|---|---|
| | | | | Normal (%) | Partly Abnormal (%) | Abnormal (%) |
| Nil | 0·5 | 17 | 16 | 0 | 18 | 82 |
| | | | 25 | 0 | 12 | 88 |
| | | | 29 | 0 | 0 | 100 |
| Sucrose (100 mg/plant) | 2·0 | 18 | 16 | 88 | 6 | 6 |
| | | | 25 | 67 | 22 | 11 |
| | | | 29 | 56 | 22 | 22 |
| Glucose (55 mg/plant) | 2·0 | 20 | 16 | 75 | 20 | 5 |
| | | | 25 | 60 | 5 | 35 |
| | | | 29 | 50 | 10 | 40 |
| Potassium sulphate (21·75 mg/plant) | 2·0 | 13 | 16 | 70 | 15 | 15 |
| | | | 25 | 31 | 7 | 62 |
| | | | 29 | 7 | 23 | 70 |
| Mannitol (55 mg/plant) | 2·0 | 16 | 16 | 0 | 0 | 100 |

The differences in expressivity, and hence of penetrance, are due firstly to slight but uncontrollable inequalities in the environment, and secondly to the differential occurrence of delayed expression of the mutant phenotype during growth. The effect of the mutation is expressed primarily on leaf growth and with most treatments a proportion of the plants may have normal formation of the first or second leaf pair while subsequent leaves become abnormal. Such reversion to the mutant phenotype suggests that the plant's requirement for a component of the substrate may exceed its rate of uptake. The frequency of this reversion is very much greater with potassium sulphate than with glucose in the substrate (Figs. 1 and 2).

The fact that potassium sulphate is almost as effective as glucose or sucrose in restoring the wild-type phenotype, at least initially, indicates that the mutant requires sugar because of its osmotic properties, and that the mutation has affected

the osmotic relations of the plant. Mannitol, on the other hand, induces full pene-trance and eventually causes the death of mutant plants. At the concentration shown in Table 1, mannitol has no pronounced effect on wild-type plants.

Plant cells have repeatedly been found to be impermeable to mannitol (Collander and Bärlund 1933; Thimann, Slater, and Christiansen 1950), so the mutant cannot require an increased osmotic pressure in the substrate. On the basis of the data on phenotype expression it appears possible that the mutant needs a source of solutes with which it may increase the internal osmotic pressure.
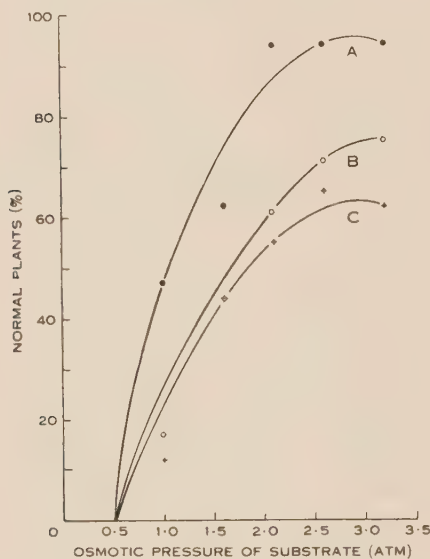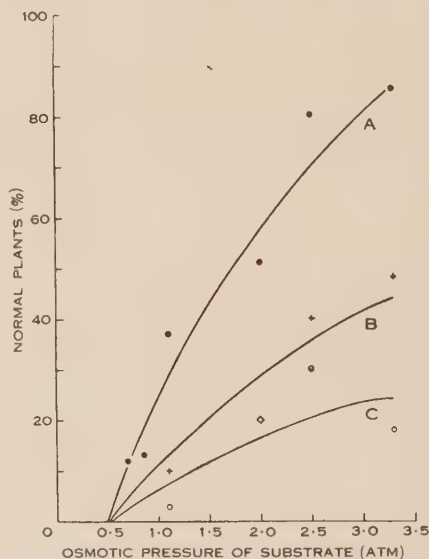


Fig. 1            Fig. 2

Fig. 1.—Percentage of phenotypically normal plants obtained from plants of mutant genotype by increasing the osmotic pressure of the substrate by the addition of glucose. A, plants 14 days old; B, 18 days old; C, 22 days old.

Fig. 2.—The same as Figure 1, but with potassium sulphate as the osmotic agent.

(ii) *Leaf and Root Growth.*—As already mentioned, one of the most pronounced effects of the mutation is to cause the leaves to be very narrow, rather short, and more or less closely curled. However, as the concentration of glucose or potassium sulphate in the medium increases, the leaves become wider and longer. The leaf curling prevented the accurate measurement of leaf lengths, but the width of the lamina of the first leaf was taken as an index of the effect of treatments upon leaf growth (Fig. 3). Leaf size and shape are closely related to normality of phenotype and, as expected, glucose is most effective in increasing leaf width, with potassium sulphate being slightly inferior. The minimum concentration of glucose that gives maximum leaf width is about 80 mg per plant corresponding to a substrate osmotic pressure of 2·6 atm. Mannitol, even at low concentration, markedly reduces the leaf width of the mutant, further confirming the inferences drawn in the previous section. The leaf width of the wild type is $4·0\pm0·25$ mm, and with equimolar

concentrations of glucose $3 \cdot 8 \pm 0 \cdot 17$, sucrose $3 \cdot 9 \pm 0 \cdot 17$, and potassium sulphate $4 \cdot 6 \pm 0 \cdot 14$ mm.

Primary root growth is not affected by the mutation but the number of secondary roots is halved (Table 2), and this in turn may retard water absorption.

TABLE 2

EFFECT OF OSMOTIC AGENTS ON ROOT GROWTH

Age of plant 17 days, temperature 23°C

| Osmotic Agent | Osmotic Pressure of Substrate (atm) | Mutant | | Wild Type | |
|---|---|---|---|---|---|
| | | Primary Root Length (mm) | Number of Secondary Roots | Primary Root Length (mm) | Number of Secondary Roots |
| Nil | $0 \cdot 5$ | $24 \cdot 8 \pm 1 \cdot 0$ | $9 \cdot 5 \pm 1 \cdot 0$ | $29 \cdot 0 \pm 0 \cdot 3$ | $18 \cdot 4 \pm 0 \cdot 5$ |
| Glucose (55 mg/plant) | $2 \cdot 0$ | $26 \cdot 9 \pm 0 \cdot 2$ | $15 \cdot 3 \pm 0 \cdot 5$ | $28 \cdot 5 \pm 0 \cdot 7$ | $18 \cdot 2 \pm 0 \cdot 8$ |
| Potassium sulphate (21·75 mg/plant) | $2 \cdot 0$ | $21 \cdot 2 \pm 0 \cdot 8$ | $14 \cdot 4 \pm 0 \cdot 8$ | $28 \cdot 0 \pm 0 \cdot 5$ | $17 \cdot 8 \pm 0 \cdot 6$ |
| Mannitol (55 mg/plant) | $2 \cdot 0$ | $27 \cdot 5 \pm 0 \cdot 6$ | $4 \cdot 9 \pm 1 \cdot 0$ | $26 \cdot 4 \pm 0 \cdot 6$ | $15 \cdot 4 \pm 0 \cdot 6$ |

The poor secondary root growth evidently results from some effect of osmotic insufficiency, for potassium sulphate is as effective as glucose in increasing root number.
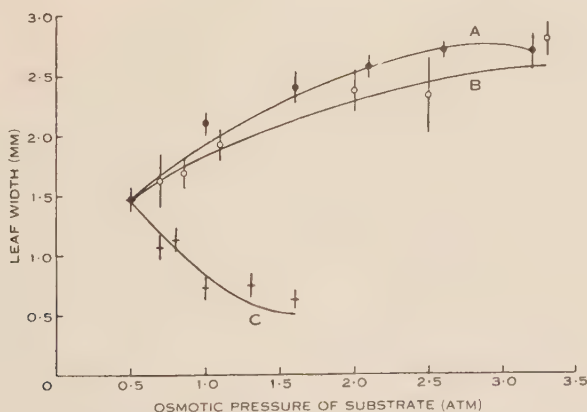


Fig. 3.—Widths of the laminae of mutant plants with increasing osmotic pressure of the medium. *A*, potassium sulphate; *B*, glucose; *C*, mannitol.

(iii) *Fertility.*—When grown on minimal medium the mutant forms numerous short pods which are completely devoid of seed. However, there is a linear relationship between the number of seeds set per plant and the osmotic pressure of the

substrate (as given by glucose) (Fig. 4). Potassium sulphate at low concentrations permits the formation of a few seeds, but at higher concentrations it causes the flower stalks to die back at the time of flowering. Even in the wild type, potassium sulphate, while allowing good vegetative growth, causes similar dying of the flower stalks and almost complete sterility (Table 3). Mannitol kills mutant plants before flowering and results in nearly complete sterilization of the wild type. Sucrose is not as effective as glucose in restoring fertility in the mutant and it is slightly inhibitory in the wild type.

Pollinated stigmas of mutant plants growing on minimal medium were squashed, stained with cotton blue in lactophenol, and examined microscopically. In contrast with the situation in wild-type plants, mutant pollen usually failed to germinate on

TABLE 3

FERTILITY OF MUTANT AND WILD TYPE GROWN ON DIFFERENT SUBSTRATES

| Osmotic Agent | Substrate Osmotic Pressure (atm) | Temperature (°C) | No. of Seeds per Plant | |
|---|---|---|---|---|
| | | | Mutant | Wild Type |
| Nil | 0·5 | 23 | 0 | 275±32 |
| | | 28 | 0 | 0 |
| Glucose (55 mg/plant) | 2·0 | 23 | 136±8 | 265±21 |
| | | 28 | 0 | 44±12 |
| Sucrose (100 mg/plant) | 2·0 | 23 | 89±11 | 266±13 |
| Potassium sulphate (21·75 mg/plant) | 2·0 | 23 | 0 | 0·8±0·6 |
| Mannitol (55 mg/plant) | 2·0 | 23 | 0 | 9±3 |

the mutant stigma or the pollen tubes grew only a short distance down the style. However, pollen from mutant plants germinates and effects fertilization on wild-type stigmas, although wild-type pollen does not germinate on mutant stigmas. A secondary effect of this mutation, therefore, is the establishment of an incompatibility system in which unilateral out-crossing is necessary for survival of the mutant gene.

## (b) Effect of Temperature

The mutant shows a pronounced change in phenotype with change in temperature. Plants in all the above experiments were grown at a uniform temperature of 23°C, in which the mutant phenotype is well expressed and viability reasonably good. At 18°C mutant plants are extremely small and die after about 14 days growth, but at 28°C differentiation is always normal although the leaves are smaller than in the wild type. Also, while wild-type plants have decreased growth as shown by dry weight at 28°C, the dry weight of the mutant is three times greater at 28 than at 23°C (Table 4). Similarly, the leaf width of the mutant growing on minimal

medium is twice as great at the higher temperature, but that of the wild type is significantly decreased (Table 5).

The high temperature (28°C) does not restore the fertility of mutant plants but even the wild type is completely sterile at this temperature (Table 3). Fertility in the wild type is partially restored by glucose, but sugars have no such effect on seed formation in mutant plants.

TABLE 4

DRY WEIGHTS OF MUTANT AND WILD TYPE

| Temperature (°C) | Mutant | | Wild Type | |
|---|---|---|---|---|
| | Number of Plants | Dry Weight (mg) | Number of Plants | Dry Weight (mg) |
| 23 | 14 | $0 \cdot 9 \pm 0 \cdot 01$ | 12 | $5 \cdot 6 \pm 0 \cdot 01$ |
| 28 | 15 | $2 \cdot 8 \pm 0 \cdot 1$ | 11 | $3 \cdot 2 \pm 0 \cdot 07$ |

### (c) Phenocopy Production

The action of glucose and potassium sulphate in restoring growth suggests that this mutation causes a low solute concentration in the cells and thus a low osmotic pressure. Attempts were made to measure directly the osmotic pressure of wild-type and mutant plants. The plasmolytic method of measuring osmotic pressure

TABLE 5

LEAF WIDTHS OF MUTANT AND WILD TYPE

| | Treatment | Leaf Width (mm) | |
|---|---|---|---|
| | | 23°C | 28°C |
| Mutant | Nil | $1 \cdot 1 \pm 0 \cdot 12$ | $2 \cdot 1 \pm 0 \cdot 12$ |
| | Glucose (55 mg/plant) | $1 \cdot 9 \pm 0 \cdot 1$ | $2 \cdot 8 \pm 0 \cdot 08$ |
| Wild type | Nil | $4 \cdot 0 \pm 0 \cdot 26$ | $3 \cdot 0 \pm 0 \cdot 11$ |
| | Glucose (55 mg/plant) | $3 \cdot 8 \pm 0 \cdot 12$ | $3 \cdot 2 \pm 0 \cdot 08$ |

is feasible only with especially favourable plant tissues, while insufficient cell sap was obtainable for cryoscopic determinations, even on a microscale. The mechanical vibration method of Virgin (1955) for the estimation of turgor pressure also proved

unsuitable. However, a very approximate estimate of osmotic pressure was obtained by observing the curvature of split petioles in a graded series of sucrose solutions. This method gave an osmotic pressure of $6 \cdot 5$ atm for wild-type tissue and $5 \cdot 0$ atm for mutant tissue, both grown on minimal medium.

As a test of the correctness or otherwise of the inference that the characteristic phenotype of the mutant resulted from an abnormally low internal osmotic pressure, an attempt was made to induce a phenocopy from the wild type. One consequence of a low osmotic pressure in the vacuolar sap is a reduced turgor pressure which is
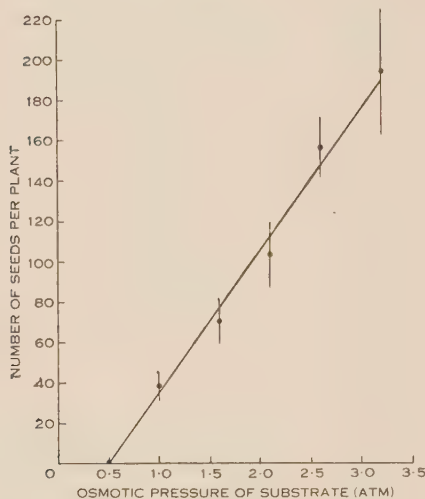


Fig. 4.
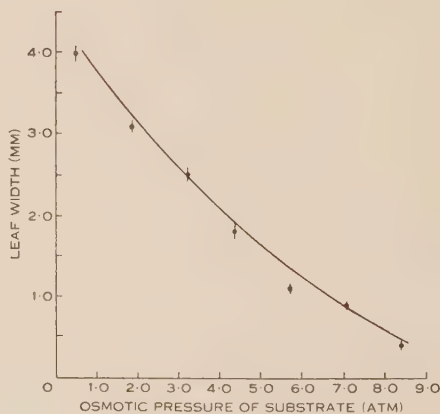
Fig. 5.

Fig. 4.—Number of seeds set by mutant plants as the osmotic pressure of the medium is increased by the addition of glucose.

Fig. 5.—Widths of the laminae of phenocopies of the mutant. Wild-type plants grown on medium containing mannitol.

equal to the difference between the osmotic pressure of the cell contents and that of the surrounding medium. Thus the internal turgor pressure (or effective osmotic pressure) of a plant may be decreased by increasing the osmotic pressure of the substrate with a substance such as mannitol to which the cell membrane is impermeable. Thus, for the production of a phenocopy of the mutant under study, wild-type plants were grown at 23°C on agar containing graded concentrations of purified mannitol.

The effect of the mannitol, even at low concentrations, was to change the same characters as the mutation affects, i.e. leaf width, cell growth, and fertility. The phenotype which most closely approached that of the mutant (at 23°C on minimal medium) was found in plants growing on 150 mg of mannitol per test tube, corresponding to an osmotic pressure of $4 \cdot 0$ atm.

Figure 5 shows that the leaf width of the wild type decreases markedly as the concentration of mannitol in the substrate increases, and therefore, as the

consequent turgor pressure of the cells decreases. The turgor pressure of the wild type must be reduced by 4·5 atm before its leaf width corresponds to that of the unsupplemented mutant.

### (d) Cell Growth

Following a description of the expression of the mutation on plant habit and tissue growth, the next logical step was an examination of cell growth. The first leaves, when mature, were fixed in Navashin's fluid, embedded in paraffin, sectioned transversely at $20\,\mu$, and stained in saffranin and fast green.

TABLE 6

EFFECT OF SUPPLEMENTS AND TEMPERATURE ON LEAF AND CELL GROWTH

Measurements and cell counts made to the midrib on the wider half of the leaf to avoid differences resulting from bilateral asymmetry. Mean cell diameter obtained by dividing the half-leaf width by the number of cells

| Treatment | Growing Temperature (°C) | Mutant | | | Wild Type | | |
|---|---|---|---|---|---|---|---|
| | | Mean Cell Diameter ($\mu$) | Number of Cells | Leaf Width to Midrib (mm) | Mean Cell Diameter ($\mu$) | Number of Cells | Leaf Width to Midrib (mm) |
| Nil | 23 | 14·8 | 25 | 0·37 | 24·1 | 101 | 2·41 |
| Nil | 28 | 27·3 | 42 | 1·15 | 14·8 | 100 | 1·48 |
| Glucose (55 mg/plant) | 23 | 14·9 | 91 | 1·36 | 23·4 | 119 | 2·79 |
| Potassium sulphate (21·75 mg/plant) | 23 | 23·2 | 65 | 1·51 | 28·5 | 112 | 3·19 |

As the leaves of the mutant often tend to be bilaterally asymmetrical, leaf width was measured only to the midrib, and the number of adjacent palisade cells in this cross section of the leaf counted. Mean cell width was obtained by dividing the number of cells into the half-leaf width.

The results of measurements of cell number, cell size, and leaf width for mutant and wild type plants grown on different substrates and at 23 and 28°C are presented in Table 6. A comparison of the numbers and sizes of the cells in the mutant and wild type on minimal medium shows that the narrow leaf width of mutant plants is caused by its having few cells which are much smaller than normal. The addition of glucose to the substrate has a very marked effect in increasing cell division, although it causes no increase in cell size. Potassium sulphate, on the other hand, results in much larger cells even in the wild type, but cell number is not so greatly increased. The different modes of action of these two substances in increasing leaf width is

more clearly seen in Figures 6 and 7. When glucose is supplied, the graph for cell number closely follows that for leaf width; but with potassium sulphate, it is the graph for cell size which approximates to the leaf-width graph.

The morphological effect of an increase in temperature is brought about through a doubling of cell size with a lesser stimulation of cell division. The expansion of wild-type cells, however, is markedly reduced. As an increase in temperature normally causes a decrease in the osmotic pressure of plant cells (Went 1955), it is assumed that the higher temperature repairs the basic biochemical deficiency in the mutant.
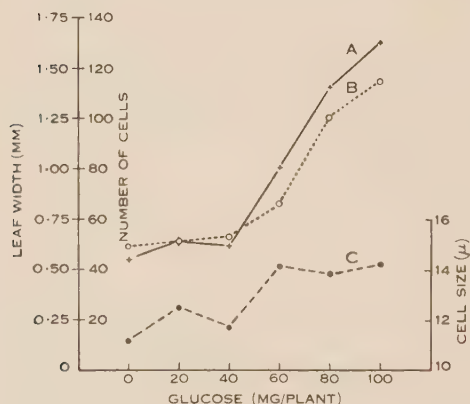


Fig. 6.—Leaf widths to the midrib, and the number and size of the cells contained therein, of mutant plants growing on increasing concentrations of glucose.  *A*, leaf width;  *B*, number of cells;  *C*, cell size.
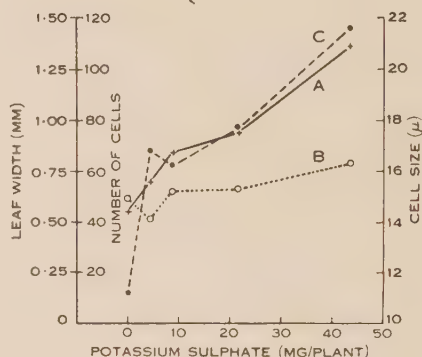
Fig. 7.—The same as Figure 6, but with potassium sulphate added to the medium.

The fact that the mutation affects both the number and size of leaf cells may seem at variance with the hypothesis that its phenotypic expression results solely from decreased osmotic pressure. However, this hypothesis has been verified by an examination of the cell behaviour of leaves of the phenocopies (wild-type plants on mannitol substrates). Here the action of mannitol, which can only be due to a lowering of internal osmotic and turgor pressure, is to decrease cell number and size as does the mutation (Fig. 8).

### (e) *Carbohydrate Metabolism and Cell Permeability*

Attempts were made to find the cause of the low osmotic pressure of cells of mutant plants. Two possibilities seemed worth investigating. Firstly, that there is a deficiency in carbohydrates causing a low cell solute concentration and a lack of energy available for cell division. Secondly, that an increased permeability of mutant cells leads to a rapid loss from the plant of both sugars and inorganic constituents.

(i) *Carbohydrate Metabolism.*—Manometric measurements of photosynthesis and respiration of whole plants using the $CO_2$ buffer system described by Pardee (1946) revealed no difference between normal and wild type.

The formation and dissolution of starch in the leaf cells was next examined. Leaf tissue was sectioned at 50 $\mu$ and stained in a solution of iodine plus potassium iodide. The chloroplasts of both mutant and wild-type plants contained abundant starch both at 23 and 28°C. No discernible difference was found in the rate of
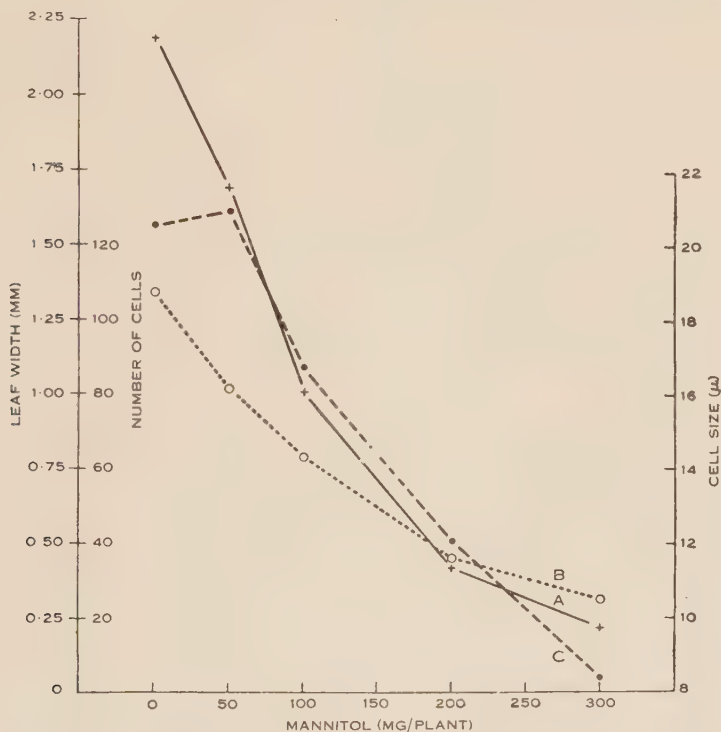


Fig. 8.—Leaf widths to the midrib, and the number and size of the cells contained therein, of wild-type plants growing on increasing concentrations of mannitol. *A*, leaf width; *B*, number of cells; *C*, cell size.

breakdown of the starch in the dark at any of the temperatures tested. An examination of the ability of mutant and wild-type leaf tissue to synthesize starch from glucose 1-phosphate was made following the method of Badenhuizen (1955). Again no consistent differences were found.

(ii) *Cell Permeability.*—For the study of permeability, the relatively large semi-transparent cells forming the stigma surface were used. Permeability to water was measured by plotting the number of cells plasmolysed against time when stigmas were immersed in a 1M solution of glucose. Further tests of permeability were made from rates of deplasmolysis of fully plasmolysed cells in 1M solutions of urea or potassium sulphate. In all experiments the behaviour of mutant cells fully conformed with that of wild-type cells.

## IV. Discussion

From the data obtained in the above experiments, the interrelated consequences of this mutation may be summarized diagrammatically. Some of the steps, difficult to measure quantitatively in this material, have been inferred from previous work, while the order of other steps has been deduced from the pattern of effect produced by supplements or temperature.

```
                        Mutant gene
                             |
                             | ←  —  —  —  —  —  — temperature
                             ↓
                    low solute concentration
                             |
                             |
                             ↓
                     lower osmotic pressure
                             |
                             |
                             ↓
             reduced water uptake ←  —  —  —  —  — mannitol
                             |
                             |
                             ↓
             ┌───────lower turgor pressure───────┐
             |                   |               |
        ←  —  —  —  —  —  |  —  —  —  —  —  |  ←  —  — glucose, sucrose
             |                   |               |
        ←  —  —  —  —  —  ←  —  —  —  —  —  |  —  — potassium sulphate
             |                   |               |
      pollen-stigma        decreased cell    reduced cell
       imbalance            expansion         division
             |                   |               |
             ↓                   └────  narrow ←─┘
     failure in pollen                 leaves
      tube growth
             |
             ↓
        sterility
```
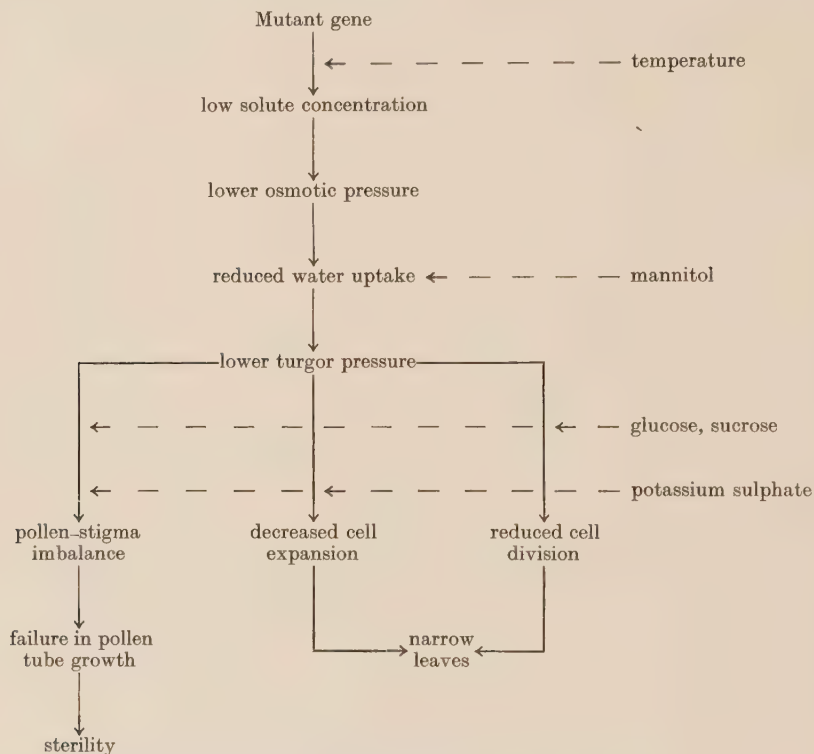
The study of this mutant is incomplete because no cause could be found for the low osmotic pressure of mutant cells. There could be at least four possible reasons for this reduced osmotic pressure:

(1) A lowered rate of metabolism or an alteration in balance between photosynthesis and respiration resulting in a lower solute level in the cell vacuoles, or insufficient energy for osmotic work.

(2) A block in starch degradation which would lock up the products of photosynthesis in an osmotically inactive form.

(3) An increase in cell membrane permeability reducing osmotic pressure through excessive leakage of solutes from the root cells.

(4) Alterations in those physical properties of the cytoplasm which are responsible for imbibition or ion retention by electrostatic and other forces.

It has not been possible to test all of these possibilities, although the first three appear to be eliminated as primary effects of the mutation by the experiments described in Section III (*e*).

The finding that both cell size and cell number were decreased in the mutant was an unexpected one, for general opinion (e.g. Burström 1951) is that osmotic pressure, acting through turgor pressure, is merely a driving force in cell expansion. Nevertheless, the reduction in the number of cells in wild-type plants grown on mannitol substrates proves that osmotic pressure, or the hydrostatic forces it affects, may also be a regulating factor in cell division.

Some explanation is needed for the different activities of glucose and potassium sulphate in increasing cell growth. If the osmotic deficiency of mutant cells resulted in a lack of energy source through exosmosis, this could be repaired by the absorption of glucose from the substrate. This rate of absorption (about 2 mg per day in plants of maximum vegetative growth) may, however, be insufficient to maintain an increased osmotic pressure. Glucose, therefore, could lead to an increase in cell division without affecting cell expansion, while potassium sulphate, nutritionally inert and more easily absorbed, would favour cell expansion. This explanation is unsatisfactory in that no account is taken of the fact that both substances are effective in restoring the fertility of the mutant.

This system of self-incompatibility but non-reciprocal cross-compatibility with wild type is partly comparable with the naturally occurring incompatibility mechanism of *Linum grandiflorum* (Lewis 1943). Both have as a causal basis the presence of unsuitable osmotic pressure differences between pollen and style. However, the disparity in osmotic pressure does not seem to be the full explanation for the incompatibility system of *Linum*, and it may not be the sole cause of sterility in the *Arabidopsis* mutant.

In its apparent deficiency in water relations, this mutant resembles the morphological mutant "cut" in *Neurospora crassa* (Kuwana 1953). This *Neurospora* mutant required, for normal hyphal growth, a relative humidity in excess of 94 per cent. It also was sensitive to the osmotic pressure of the medium, but, unlike the *Arabidopsis* mutant, it gave a decreased rate of growth as compared with the wild type when the osmotic pressure was raised.

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AN OSMOTIC MUTANT OF ARABIDOPSIS THALIANA



Mutant plants, 26 days old, growing on minimal medium with and without added sucrose. *1*, no sucrose; *2*, 20 mg sucrose; *3*, 50 mg sucrose; *4*, 100 mg sucrose.

# NUTRIENT INTERACTIONS AND DEFICIENCY DIAGNOSIS IN THE LETTUCE

## V. POTASSIUM CONTENT AND RESPONSE TO POTASSIUM

### By A. E. Grant Lipp* and D. W. Goodall†

*Summary*

Lettuce plants grown in sand culture, and receiving nitrogen, phosphorus, and potassium at five levels in all combinations, were analysed at different stages of growth for these elements. An attempt was made to relate these analytical data to the subsequent response (in dry matter production) shown by the plants when a further amount of potassium was supplied.

The concentration of potassium in the plant dry matter was increased by potassium supply, decreased by nitrogen and in some instances by phosphorus supply. Interactions among the nutrients were not very marked. Differences in potassium uptake at different levels of supply were already clear-cut at 11 days from sowing.

The response to potassium could be forecast from a knowledge of the potassium content of the plant at different stages; for analyses at 37 and 44 days, this relationship was markedly curvilinear. At 29 days knowledge of the phosphorus content enabled the precision of the forecast to be increased; at 37 days knowledge of the nitrogen content was more useful, while at 44 days neither of the other nutrients contributed significantly to the forecast. Little benefit could be obtained from analysing particular organs rather than the entire aerial parts.

## I. INTRODUCTION

This paper is the last of a series describing a sand-culture experiment designed to find the best way of using the nitrogen, phosphorus, and potassium content of young lettuce plants as an index of their probable response to additional supplies of these nutrients. Plants varying greatly in mineral status were obtained by treatment with each nutrient at five levels, in all combinations. These plants were sampled for analysis, and the growth responses to further applications of fixed amounts of the nutrients were determined. Previous papers have dealt with the effects of nutrient treatments on dry weight (Goodall, Grant Lipp, and Slater 1955) and water content (Goodall, Slater, and Grant Lipp 1957), and with the content of nitrogen and phosphorus particularly in relation to the response to these nutrients (Slater and Goodall 1957; Grant Lipp and Goodall 1958). The present paper will deal similarly with the potassium data.

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## II. Methods

The cultural technique, the treatments, and the sampling and harvesting procedure have been described in earlier papers, so all that is now necessary is an account of the method of analysis for potassium.

Potassium was determined by a modification of Amdur's (1940) method on aqueous extracts of the dried plant material. An aliquot containing $0 \cdot 1$–$0 \cdot 35$ mg potassium was decolorized with 100-volume hydrogen peroxide and evaporated to dryness. The dipicrylamine reagent (Williams 1941) was added to the dry residue and allowed to stand for 45 min. It was then diluted to standard volume and the colour intensity measured.

The data for some harvests were incomplete through death of plants or inadequacy of material for analysis. These gaps were filled by "missing plot" methods before statistical analysis; the methods are detailed in the original thesis (Grant Lipp 1952), where a fuller presentation of the results may also be found. Data for separate organs (older and younger leaves, midribs, and laminae), and for the final harvest at 98 days, were so incomplete that missing plot treatment would not have been justified. These results are accordingly not tabulated though reference is made to them wherever appropriate.
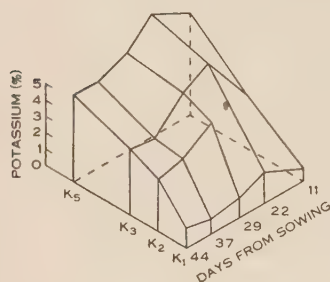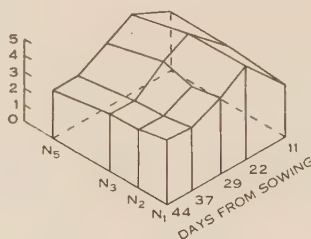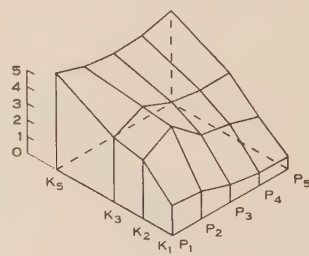


Fig. 1                          Fig. 2                          Fig. 3

Figs. 1 and 2.—Effect of potassium supply (Fig. 1) and nitrogen supply (Fig. 2) on potassium content at different stages of development.

Fig. 3.—Interaction effects of potassium and phosphorus supply on potassium content at 44 days.

## III. Results

### (a) Potassium Content as Affected by Treatments

Table 1 contains analyses of variance of the data for potassium content at each harvest, expressed as per cent. of dry matter. The mean values corresponding to significant effects are shown in Table 2, and some of the results are presented in the form of solid diagrams (Figs. 1–3).

The mean potassium content of the tissue increased abruptly during the first 3 weeks of development. The seed contained only $0 \cdot 74$ per cent., while by 11 days it had risen to an average of $3 \cdot 4$ per cent., and by 22 days to $5 \cdot 3$ per cent. Thereafter it declined until 37 days, when a steady level of $3 \cdot 4$ per cent. had been reached.

(i) *Mean Effects of Initial Treatments.*—Potassium supply affected the potassium content of the plants throughout the experiment (Fig. 1). At the earliest

TABLE 1

TOTAL POTASSIUM CONTENT (PER CENT. OF DRY MATTER) AS AFFECTED
BY INITIAL TREATMENTS: ANALYSIS OF VARIANCE

| Days from Sowing | Source of Variation | Degrees of Freedom | Mean Square |
|---|---|---|---|
| 11 | N | 2 | 0·317 |
|  | P | 2 | 0·192 |
|  | K | 2 | 65·590** |
|  | N × P | 4 | 0·390 |
|  | N × K | 4 | 0·217 |
|  | P × K | 4 | 0·183 |
|  | Error | 3 | 1·972 |
| 22 | N | 2 | 2·318* |
|  | P | 2 | 0·419 |
|  | K | 2 | 80·887*** |
|  | N × P | 4 | 0·388 |
|  | N × K | 4 | 0·290 |
|  | P × K | 4 | 0·608 |
|  | Error | 8 | 0·464 |
| 29 | N | 3 | 2·725 |
|  | P | 4 | 1·138 |
|  | K | 3 | 95·076*** |
|  | N × P | 12 | 0·425 |
|  | N × K | 9 | 0·802 |
|  | P × K | 12 | 0·908 |
|  | Error | 31 | 1·173 |
| 37 | N | 3 | 2·773* |
|  | P | 4 | 1·278 |
|  | K | 3 | 64·887*** |
|  | N × P | 12 | 0·942 |
|  | N × K | 9 | 1·116 |
|  | P × K | 12 | 0·647 |
|  | Error | 28 | 0·801 |
| 44 | N | 3 | 3·418*** |
|  | P | 4 | 3·152*** |
|  | K | 3 | 58·616*** |
|  | N × P | 12 | 0·202 |
|  | N × K | 9 | 0·515 |
|  | P × K | 12 | 0·792** |
|  | Error | 30 | 0·254 |

* $P$: 0·01–0·05.   ** $P$: 0·001–0·01.   *** $P < 0.001$.

harvest, only 11 days after sowing, the differences were already well marked. From 29 days, the first occasion on which plants receiving $K_2$ were analysed, the main increase in potassium content was between $K_1$ and $K_2$ ($K_1$ received no added

potassium). Increase in potassium supply beyond $K_2$ had a smaller, though definite, effect on potassium content. This effect became greater at succeeding harvests. The rather scattered data for 98 days suggest that the differences in potassium content between different treatments had by that time become greater.

Except at 11 days, increase in nitrogen supply led to decrease in potassium content (Fig. 2), significant at 22, 37, and 44 days, and similar but non-significant at 29 days. This effect was more marked between $N_3$ and $N_5$ than at lower levels of nitrogen supply. The decrease in potassium content with nitrogen supply was still apparent at 98 days.

TABLE 2

POTASSIUM CONTENT (PER CENT. OF DRY MATTER) AS AFFECTED BY INITIAL TREATMENTS

11 *Days from Sowing*

Mean Effects at Different Levels of Initial Treatments

| | | | | | |
|---|---|---|---|---|---|
| $N_1$ | 3·19 | $P_1$ | 3·24 | $K_1$ | 0·78 |
| $N_3$ | 3·47 | $P_3$ | 3·49 | $K_3$ | 3·26 |
| $N_5$ | 3·55 | $P_5$ | 3·49 | $K_5$ | 6·17 |

22 *Days from Sowing*

Mean Effects at Different Levels of Initial Treatments

| | | | | | |
|---|---|---|---|---|---|
| $N_1$ | 5·66 | $P_1$ | 5·25 | $K_1$ | 1·92 |
| $N_3$ | 5·59 | $P_3$ | 5·58 | $K_3$ | 6·55 |
| $N_5$ | 4·75 | $P_5$ | 5·17 | $K_5$ | 7·53 |

29 *Days from Sowing*

Mean Effects at Different Levels of Initial Treatments

| | | | | | |
|---|---|---|---|---|---|
| $N_1$ | 4·59 | $P_1$ | 3·94 | $K_1$ | 1·21 |
| $N_2$ | 4·26 | $P_2$ | 4·46 | $K_2$ | 4·76 |
| $N_3$ | 4·78 | $P_3$ | 4·41 | $K_3$ | 5·61 |
| | | $P_4$ | 4·65 | | |
| $N_5$ | 3·94 | $P_5$ | 4·49 | $K_5$ | 5·99 |

37 *Days from Sowing*

Mean Effects at Different Levels of Initial Treatments

| | | | | | |
|---|---|---|---|---|---|
| $N_1$ | 3·79 | $P_1$ | 3·56 | $K_1$ | 0·93 |
| $N_2$ | 3·49 | $P_2$ | 3·69 | $K_2$ | 3·56 |
| $N_3$ | 3·40 | $P_3$ | 3·22 | $K_3$ | 3·84 |
| | | $P_4$ | 3·51 | | |
| $N_5$ | 2·89 | $P_5$ | 2·99 | $K_5$ | 5·24 |

TABLE 2 (Continued)

44 Days from Sowing

Mean Effects at Different Levels of Initial Treatments

| | | | | | |
|---|---|---|---|---|---|
| $N_1$ | 3·87 | $P_1$ | 3·76 | $K_1$ | 1·22 |
| $N_2$ | 3·52 | $P_2$ | 4·04 | $K_2$ | 3·19 |
| $N_3$ | 3·47 | $P_3$ | 3·27 | $K_3$ | 4·01 |
| | | $P_4$ | 3·05 | | |
| $N_5$ | 2·87 | $P_5$ | 3·05 | $K_5$ | 5·30 |

Interactions between Initial Treatments

| | $P_1$ | $P_2$ | $P_3$ | $P_4$ | $P_5$ |
|---|---|---|---|---|---|
| $K_1$ | 1·82 | 1·65 | 1·09 | 0·78 | 0·76 |
| $K_2$ | 3·54 | 4·55 | 3·04 | 2·67 | 2·14 |
| $K_3$ | 3·87 | 4·74 | 4·00 | 3·72 | 3·74 |
| $K_5$ | 5·78 | 5·20 | 4·97 | 5·02 | 5·54 |

Phosphorus supply, which had very marked effects on the plants in all other respects, had remarkably little influence on potassium content. At 44 days a decrease in potassium content with phosphorus supply became significant—a decrease already suggested by the values for 37 days. Such data as are available for 98 days indicate that this tendency had by that time become more marked.

(ii) *Interactions among Initial Treatments.*—On the whole, the other nutrients played little part in determining the influence of potassium supply on potassium content. At 44 days, to be sure, there was a significant interaction between potassium and phosphorus (Fig. 3), the effect of phosphorus in reducing potassium content being apparent only at the lower ($K_1$ and $K_2$) levels of potassium supply. There was no indication of such an interaction at 37 days, and the data for 98 days are insufficient to enable one to judge whether it still existed at that time.

Interactions between nitrogen and potassium and between nitrogen and phosphorus were at no time significant.

(iii) *Effects of Sub-treatment.*—Where no potassium had been supplied initially, the potassium sub-treatment caused a very substantial increase in potassium content—from *c.* 0·8 to *c.* 3·7 per cent. in otherwise comparable plants. The potassium content of these plants was in fact nearly double that of plants which had received the same amount of potassium at the time of planting (treatment $K_2$). At higher initial levels of potassium supply, the effect of supplementary potassium on its concentration in the plant was rather irregular.

Supplementary nitrogen seems to have reduced the potassium content where the initial nitrogen supply was low, but not in treatments with $N_4$ or $N_5$. Supplementary phosphorus had no effects that can be clearly recognized.

## (b) Potassium Uptake

From the data for dry weight and per cent. potassium, it was possible to calculate the total quantity of potassium in the aerial parts of the plants at successive stages of development, and under the various treatments. For convenience, we have termed this quantity "potassium uptake". These calculations have been performed for the 1, 3, and 5 levels only of each nutrient.

The course of potassium uptake with the three levels of potassium supply is shown in Figure 4. By 11 days, the amount of potassium taken up already differed markedly with potassium supply, the amount in the seed having been multiplied
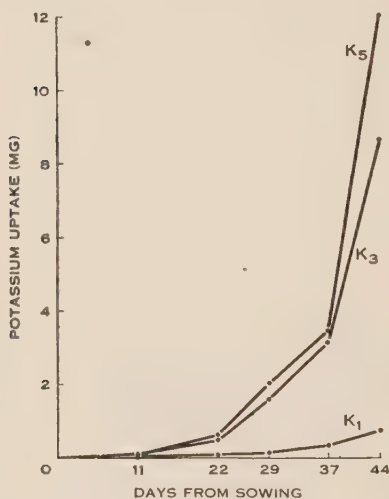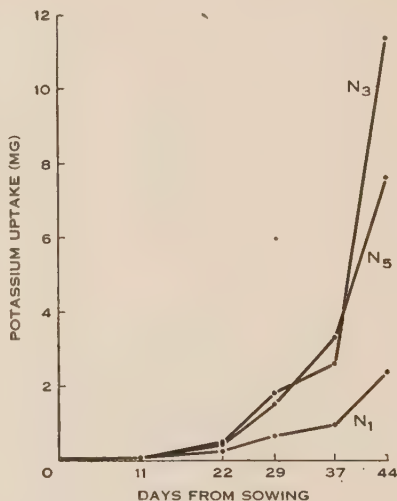


Fig. 4                Fig. 5

Figs. 4 and 5.—Potassium uptake (mg per plant) as affected by potassium supply (Fig. 4) and nitrogen supply. (Fig. 5)

by eight ($K_3$) or 13 ($K_5$) where the medium contained added potassium. Even where no potassium had been added ($K_1$), appreciable amounts of potassium were taken up after this period, one plant containing as much as 12 mg at 98 days. This was doubtless derived from impurities in the sand. At higher levels of potassium supply, by no means all the nutrient supplied was absorbed by the plants. In plants receiving the $K_3$ level of supply, the proportion taken up was little more than one-fifth, and at the $K_5$ level considerably less.

Initial supplies of both nitrogen and phosphorus affected potassium uptake (Figs. 5 and 6). The enhanced growth resulting from increase in phosphorus supply was accompanied by increased uptake. Increase in nitrogen supply from $N_1$ to $N_3$ caused increased uptake. Increasing the nitrogen supply to $N_5$ had only a slight effect on uptake at the first four harvests. At 44 days, however, when the adverse effect of the highest nitrogen level on growth had become established, it decreased uptake.

## (c) *Relationship between Growth Response to Potassium and Chemical Composition*

The primary purpose of this investigation was to study the way in which the concentration of nitrogen, phosphorus, and potassium in the plant tissue could be used to predict the responses of the plants to these three nutrients severally. The calculation of multiple regressions serves to indicate how close the relation-
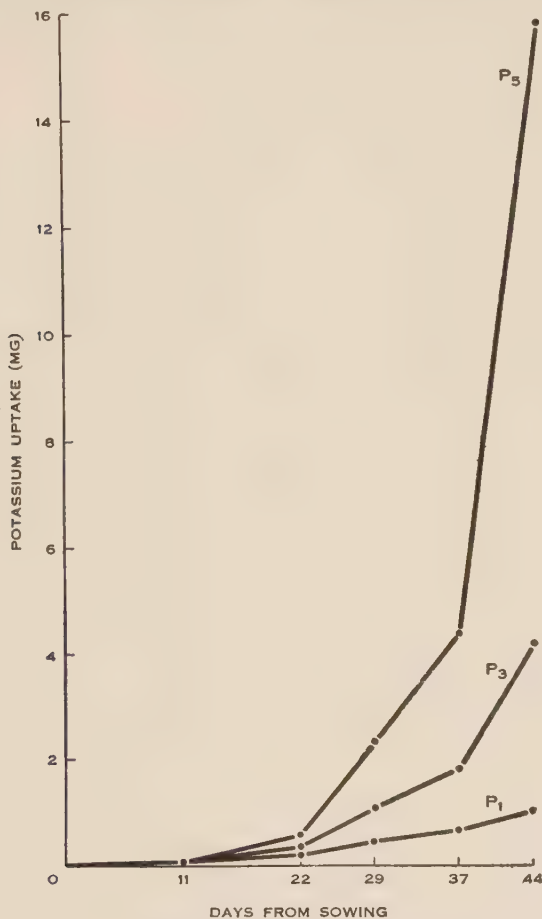


Fig. 6.—Potassium uptake (mg per plant) as affected by phosphorus supply.

ship was between the response on the one hand, and the content of the three nutrients in different plant parts, and at different stages of development, on the other. As the dependent variable ($y$), the difference in dry weight (in mg) at 98 days between the plant subjected to potassium sub-treatment and its control was used, adjusted for the difference in size recorded for these two plants before application of the sub-treatment (Grant Lipp 1952). The independent variables ($x_N$, $x_P$, $x_K$) were the nutrient contents expressed as per cent. of dry matter. The regression coefficients are therefore all in terms of milligrams response per unit per cent. change in composition.

The responses for the various combinations of initial treatments are tabulated in Table 3. In Table 4 are given the results of the regression analyses, using the data for the composition of entire aerial parts at different stages of development. It will be noted that only 67 sets of data were available; this is because, although material from 80 initial treatments was analysed chemically, data for one or other of the variables were missing in 13 of these. At 11 and 22 days the analytical data covered too few treatments to justify regression analysis.

TABLE 3

GROWTH RESPONSE TO POTASSIUM (IN MG DRY MATTER PER PLANT)

| Nitrogen Levels | Potassium Levels | Phosphorus Levels | | | | |
|---|---|---|---|---|---|---|
| | | $P_1$ | $P_2$ | $P_3$ | $P_4$ | $P_5$ |
| $N_1$ | $K_1$ | + 39 | −260 | + 264 | − 42 | + 847 |
| | $K_2$ | + 3 | − 56 | +1363 | + 228 | − 597 |
| | $K_3$ | + 11 | −188 | + 39 | + 226 | − 182 |
| | $K_5$ | − 10 | − 82 | − 213 | − 103 | − 111 |
| $N_2$ | $K_1$ | + 63 | +235 | — | +1246 | +2890 |
| | $K_2$ | −139 | +328 | + 597 | −1017 | − 122 |
| | $K_3$ | + 1 | −134 | −1468 | −1621 | − 57 |
| | $K_5$ | + 38 | + 22 | + 24 | + 93 | − 445 |
| $N_3$ | $K_1$ | 0 | + 26 | + 204 | +2876 | +3639 |
| | $K_2$ | + 16 | −170 | − 826 | — | +1865 |
| | $K_3$ | − 27 | − 47 | − 826 | −3270 | — |
| | $K_5$ | −159 | − 1 | −1082 | −2115 | +3413 |
| $N_5$ | $K_1$ | + 36 | + 30 | + 210 | +3903 | +1166 |
| | $K_2$ | − 94 | + 22 | + 114 | +1440 | +1490 |
| | $K_3$ | + 17 | − 7 | − 33 | − 796 | −1023 |
| | $K_5$ | − 3 | + 4 | — | + 698 | − 401 |

By 29 days the regression had reached a high level of significance. The most important component in this multiple regression was that on potassium content; phosphorus took second place, while the contribution of nitrogen to the estimation of response was negligible at this stage. The regression equation may accordingly be written:

$$y = 323 \cdot 6 + 1897 \cdot 1\ x_P - 189 \cdot 8\ x_K.$$

At 37 days, the situation had changed, in that the simple regressions on each of the three independent variables were about equal in significance, that on nitrogen content being slightly greater than the other two. However, that part of the regression due to potassium formed a significant addition to the regression on the other independent variables, whereas this was not true of nitrogen or phosphorus,

though either taken separately improved the precision of prediction of response over that obtainable from potassium content alone. In other words, for optimum prediction of response from analyses at 37 days potassium data are needed, together with either phosphorus or nitrogen data, but not both. This is a reflection of the closeness with which phosphorus and nitrogen contents were associated in this experiment. The regression equations, which may be regarded as alternatives, are:

$$y = -829 \cdot 3 + 507 \cdot 2 \, x_N - 160 \cdot 1 \, x_K,$$

$$y = 129 \cdot 7 + 2189 \cdot 7 \, x_P - 170 \cdot 9 \, x_K.$$

TABLE 4

ANALYSIS OF VARIANCE OF THE REGRESSION OF RESPONSE ON COMPOSITION OF ENTIRE AERIAL PARTS
The mean square for reduction due to regression was tested in each case against the residual mean square for that regression. The effect of reducing predicting variables from three to two (or one), given by the difference in the sum of squares due to regression divided by one (or two) degrees of freedom, can be tested against the residual mean square fitting N, P, and K

| Reduction due to Regression on: | Degrees of Freedom | 29 Days | | 37 Days | | 44 Days | |
|---|---|---|---|---|---|---|---|
| | | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ |
| N, P, and K | 3 | 2140 | 713*** | 1740 | 580** | 1782 | 594** |
| N and P | 2 | 1182 | 591** | 1330 | 665** | 1529 | 765** |
| N and K | 2 | 1762 | 881*** | 1658 | 829** | 1635 | 817** |
| P and K | 2 | 2111 | 1056*** | 1555 | 778** | 1620 | 810** |
| N alone | 1 | 666 | 666* | 1193 | 1193** | 1254 | 1254** |
| P alone | 1 | 1172 | 1172** | 1015 | 1015** | 1233 | 1233** |
| K alone | 1 | 1482 | 1482*** | 1039 | 1039** | 1128 | 1128** |
| Residue fitting N, P, and K | 63 | 6357 | 101 | 6754 | 107 | 6716 | 107 |

*P: 0·01–0·05.    **P: 0·001–0·01.    ***P < 0·001.

At 44 days, the same trend had continued further. Again, the simple regression on each independent variable separately was highly significant. The addition of either nitrogen or phosphorus data enabled the prediction based on potassium content to be improved, but information on both was unnecessary. The potassium contribution to the regression was of doubtful significance—either nitrogen or phosphorus data alone enabled almost as good a prediction of response to be made as the combination of one of these with the potassium data. Using nitrogen or phosphorus data alone, the regression equations are:

$$y = -1528 \cdot 7 + 661 \cdot 6 \, x_N,$$

$$y = -554 \cdot 1 + 3087 \cdot 9 \, x_P.$$

If potassium data are added, these become:

$$y = -480 \cdot 0 + 473 \cdot 4 \ x_N - 161 \cdot 2 \ x_K,$$

$$y = 225 \cdot 3 + 2195 \cdot 9 \ x_P - 162 \cdot 5 \ x_K.$$

At 44 days, in the larger plants the older and younger leaves had been separated, and the former divided into midribs and laminae. In many cases these were analysed separately, making possible a comparison of the value of different types of sample of plant material for the prediction of response from chemical composition at this

TABLE 5

ANALYSIS OF VARIANCE OF THE REGRESSION OF RESPONSE ON COMPOSITION OF DIFFERENT PLANT
PARTS AT 44 DAYS

The mean square for reduction due to regression was tested in each case against the residual mean square for that regression. The effect of reducing predicting variables from three to two (or one), given by the difference in the sum of squares due to regression divided by one (or two) degrees of freedom, can be tested against the residual mean square fitting N, P, and K

| Reduction due to Regression on: | Degrees of Freedom | Laminae of Older Leaves | | Midribs of Older Leaves | | Younger Leaves | | Entire Aerial Parts | |
|---|---|---|---|---|---|---|---|---|---|
| | | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ | Sum of Squares $\times 10^{-4}$ | Mean Square $\times 10^{-4}$ |
| N, P, and K | 3 | 2223 | 741* | 2531 | 844** | 2071 | 690* | 2272 | 757* |
| N and P | 2 | 2047 | 1024 | 2331 | 1165** | 1223 | 611 | 1989 | 994* |
| N and K | 2 | 1521 | 761 | 1709 | 854* | 1787 | 893 | 1639 | 820* |
| P and K | 2 | 2221 | 1110 | 2357 | 1179** | 1936 | 968 | 2272 | 1136 |
| N alone | 1 | 717 | 717 | 1266 | 1266* | 309 | 309 | 850 | 850* |
| P alone | 1 | 2042 | 2042** | 2028 | 2028** | 1112 | 1112* | 1989 | 1989** |
| K alone | 1 | 1229 | 1229* | 1075 | 1075* | 1787 | 1787** | 1324 | 1324* |
| Residue fitting N, P, and K | 26 | 4878 | 188 | 4572 | 176 | 5031 | 194 | 4828 | 186 |

*P: 0·01–0·05.          **P: 0·001–0·01.

stage. The results of the regression analyses are presented in Table 5; included in the table are regressions on the composition of the entire aerial parts based on the same range of initial treatments as those for the separated organs—excluding, that is, the smaller plants, and thus most of those with low phosphorus treatments. It will be noted that the exclusion of these plants has had the effect of reducing considerably the association between nitrogen and phosphorus contents as compared with the more complete set of data used for Table 4.

In Table 5, a noteworthy difference appears between the younger leaves and the older ones (both midribs and laminae). In the former, potassium content provides the largest contribution to the regression—in fact the other components are non-significant. In the older leaves, phosphorus contributes most to the regression,

and the additional variance accounted for by potassium and nitrogen in the multiple regression, or either separately, is non-significant. The multiple regression variances for the different parts of the plant do not differ greatly. The data for the younger leaves lead to the following regression equation:

$$y = 2135 \cdot 8 - 723 \cdot 4 \, x_K.$$

The corresponding expression for the midribs of the older leaves is:

$$y = 1149 \cdot 2 + 7338 \cdot 0 \, x_P,$$

and for their laminae:

$$y = 1260 \cdot 8 + 5377 \cdot 3 \, x_P.$$

## IV. DISCUSSION

In this experiment potassium supply had a very marked effect on the potassium content of the plants. The potassium available in the sand used was far from negligible, but it amounted to only a small fraction of the lowest dose added. Potassium supply in excess of this smallest dose ($K_2$) had rather little effect on growth (Goodall, Grant Lipp, and Slater 1955), but increased the potassium content considerably at the later harvests, thus providing a clear instance of luxury consumption. Another reflection of luxury consumption of potassium is the effect of nitrogen and phosphorus supply in reducing potassium content. This effect of phosphorus, though, is not nearly as marked as one might expect from its pre-

TABLE 6

CURVILINEAR REGRESSION OF RESPONSE TO POTASSIUM ON POTASSIUM CONTENT
AT DIFFERENT TIMES

|  | Degrees of Freedom | Mean Square $\times 10^{-3}$ | | |
|---|---|---|---|---|
|  |  | 29 Days | 37 Days | 44 Days |
| Linear regression | 1 | 19207*** | 14266*** | 16060*** |
| Parabolic term | 1 | 2996 | 9911** | 16373** |
| Error | 68 | 1087 | 1047 | 926 |

**$P$: $0 \cdot 01$–$0 \cdot 001$.    ***$P < 0 \cdot 001$.

dominant effect on growth in this experiment—only at 44 days could such an effect be detected with certainty.

As with other nutrients, the responses to potassium added half way through development were related to the potassium content of the plants at that time and earlier. But this relationship depended on the content of nitrogen and phosphorus, and at 44 days it appeared that the content of one or other of these nutrients was sufficiently informative on the potassium response to enable information as to the potassium content itself to be dispensed with.

For nitrogen, but not for phosphorus, the relationship between content and response proved to be curvilinear. This was again tested in the case of potassium, with the results in Table 6. It will be seen that the curvilinearity is very marked for the harvests at 37 and 44 days. This observation led to the surmise that the curvilinear relationship might perhaps be independent of the control of other nutrients. This proved to be true for 44 days (Table 7), but not for 37 days, where the nitrogen content contributed significantly to the relationship.

TABLE 7

REGRESSION OF RESPONSE TO POTASSIUM ON NUTRIENT CONTENT

|  | Degrees of Freedom | Mean Square $\times 10^{-3}$ | |
| --- | --- | --- | --- |
|  |  | 37 Days | 44 Days |
| Quadratic regression on potassium content | 2 | 12089*** | 16212*** |
| Regression on nitrogen content, potassium constant | 1 | 5449* | 775 |
| Regression on phosphorus content, potassium and nitrogen constant | 1 | 1 | 250 |
| Error | 66 | 997 | 939 |

*P: 0·01–0·05.        ***P<0·001.

The curvilinearity of the response–content relationship for potassium (as also for nitrogen) agrees with Lundegårdh's (1941, 1943, 1951) findings for oats. In his case, however, this relationship depended on the phosphorus status of the plants, which was true for lettuce only at the 29-day stage.

The regression equations derived from these analyses are:
For 37 days:
$$y = 166 - 686\,x_K + 70\,x_K^2 + 476\,x_N.$$
For 44 days:
$$y = 2686 - 1508\,x_K + 184\,x_K^2.$$

The values for potassium content corresponding with zero response are given in Table 8, and the regression curves are shown in Figures 7–9.

The general picture presented by this series of papers serves to confirm the value of plant analysis in forecasting responses to fertilizer treatments, but to emphasize the complexity of the forecasting process. It is clear that the particular results obtained—especially on interactions—have been somewhat coloured by the particular range of nutritional conditions obtained in the experimental plants. Responses to phosphorus, for instance, were much more marked than those to potassium; and this may well be linked with the greater importance of other nutrients in determining potassium responses. It is clear that the results will also depend on the quantity of nutrient to which the response is measured—that is, on

the sub-treatments applied. The larger the sub-treatment, the more might the response be expected to depend on the status of the plant in respect of other nutrients.

TABLE 8

LIMITING POTASSIUM CONTENT CORRESPONDING WITH ZERO RESPONSE
TO POTASSIUM

| Days from Sowing | Phosphorus (%) | Nitrogen (%) | Limiting Potassium Content (%) |
|---|---|---|---|
| 29 | 0·1 | | 2·71 |
| | 0·2 | | 3·70 |
| | 0·3 | | 4·70 |
| 37 | | 1·0 | 1·05 |
| | | 2·0 | 2·06 |
| | | 3·0 | 3·79 |
| 44 | | | 2·62 |

Lundegårdh's conclusion that the relationship of response to nutrient content was curvilinear has been confirmed for nitrogen and potassium in these studies,
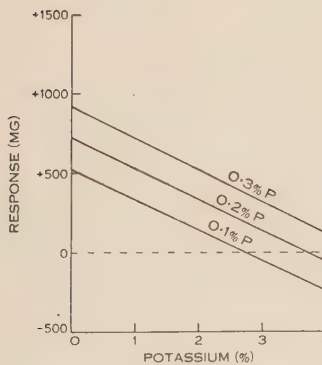


Fig. 7.
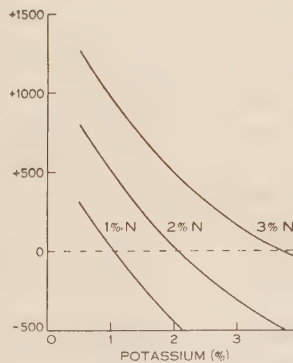
Fig. 8.
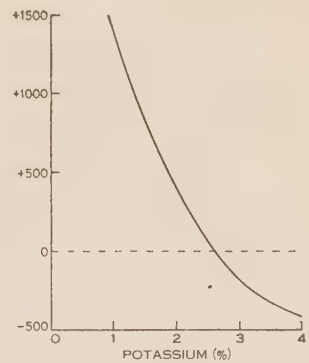
Fig. 9.

Fig. 7.—Relation between response to potassium and potassium content at 29 days, at different phosphorus levels.

Fig. 8.—Relation between response to potassium and potassium content at 37 days, at different nitrogen levels.

Fig. 9.—Relation between response to potassium and potassium content at 44 days.

though not for phosphorus. He claimed (1941) that the relationship was hyperbolic:

$$ay = b/x^c,$$

where $y$ is response, $x$ nutrient content, and the other terms constants, and he

obtained reasonable graphical agreement, though no formal test was performed. The present data could clearly not be fitted by a function of this type, for there was a considerable number of negative responses, some quite considerable. A parabolic expression, as used here, is admittedly arbitrary, but will serve until theoretical considerations can suggest a well-founded alternative.

In no case did limitation of the analyses to particular chemical fractions or particular parts of the plant greatly improve the precision with which response could be forecast. Earlier suggestions that older organs, or conducting tissue, or labile fractions indicate the nutrient status of the plant more accurately than analysis of the entire shoot could not be substantiated.

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# TRANSIENT BIOELECTRIC POTENTIALS PRODUCED BY ELECTRICALLY STIMULATED BEAN ROOTS

By I. S. Jenkinson*

*Summary*

It is shown that following electric stimulation, bean roots immersed in a weakly conducting salt solution produce transient bioelectric potentials characteristic of the type of electric stimulus applied.

The transient potentials caused by constant-amplitude alternating voltages are shown to depend uniquely on the number of cycles applied, irrespective of the frequency, provided that a sufficient amount of electric charge ($10^{-4}$ C approximately) is passed through the plant during stimulation. The plant adapts itself to the stimulus applied so that the root's potential pattern is disturbed less after the application of a large number of alternating voltage cycles than after relatively few cycles.

The transients resulting from the application of direct voltage are found to depend to some extent on the voltage polarity. However, the transient potentials caused by direct voltages of opposite polarity yield a common component in close agreement with that found for a small number of cycles of alternating voltage. The same minimum charge as for alternating voltage is required for maximum stimulation.

## I. Introduction

In an earlier paper, Scott (1957) has described spontaneous oscillatory potentials and also transient potentials following stimulation, observed at points adjacent to a bean root immersed in a weakly conducting salt solution. It was suggested that such oscillatory potentials could be explained in terms of a feedback system of control acting between certain functional variables of the biological system, including the electric field.

Since the spontaneous oscillations occur infrequently, attention has been directed to a more detailed study of transient potentials which frequently exhibit overshoots and damped oscillations before the potentials return to the steady state again. These transients are observed to result from such treatments as mechanical stimulation, sudden changes in salt concentration of the bathing solution, addition of indoleacetic acid to the bath, and exposure of the root to air for a short time.

A more readily controlled stimulus consists in the application of electric voltage between the cotyledons and the plant's bathing solution. In this paper correlations between different electric stimuli and the transient bioelectric fields occasioned on removal of such stimuli, are described. It is thought that from a study of such correlations, knowledge of the mechanism of initiation of oscillatory potentials produced by plant roots in an unchanging environment may be revealed.

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In this paper it is shown that the transient potential pattern of the root observed immediately after alternating voltage stimulation depends only on the number of cycles applied, irrespective of the alternating voltage frequency, provided that a sufficient quantity of electricity is passed through the plant during stimulation. The form of the relation is such that the bioelectric field of the plant root is disturbed less after a large number of alternating voltage cycles than. after relatively. few cycles. This indicates that the plant adapts itself to the alternating voltage, the extent to which it does so being determined solely by the number of voltage cycles to which it has been subjected.

## II. Experimental Material and Methods

The material used in the experiments described in this paper was a Long Pod variety of the broad bean, *Vicia faba L.*, which was grown in continuously circulated and aerated tap-water at 25°C. Plants 2–3 days old with roots about 4 cm long were used in most experiments.

The plant under investigation was mounted vertically in the measuring tank with the root, but not the cotyledons, immersed in a bathing solution of $10^{-4}$N KCl. The cotyledons in contact with moist cotton wool were held securely by an insulated stainless-steel clamp which acted also as one of the electrodes for the application of electric voltage stimulus, the other electrode being immersed a few centimetres below the root tip in the bathing solution.

Bioelectric potentials were measured by means of probes consisting of lengths of transparent "Nylex" tubing (2-mm bore), the ends of which were placed close to the surface of the root at various points along its immersed length. The other end of each tube dipped into an insulated plastic cup, the cups and tubes being filled with the same solution as that of the bath. Tubes came from five points near the plant while a sixth came from a distant point in the bath thus acting as a reference zero of potential. Each of these cups was connected in turn via a calomel half-cell to a six-channel automatic recording apparatus described by Scott (1957). A recording-chart speed of 3 in./hr was used throughout.

While the external voltage was applied to the plant, the electrometer grid was earthed. On removal of the applied voltage the electrometer was switched in immediately to record the transient potentials of the plant root. Voltages, either direct or alternating, were applied between the two stainless-steel electrodes, a microammeter being included in the circuit to record the total current passed through the plant.

Direct voltages were supplied by dry cells, the applied voltage stimulus being referred to as plus or minus according as the cotyledons were made positive or negative with respect to the electrode in the bathing solution. Voltages of 9 and 18 V, producing total currents through the plant of 100–250 $\mu$A, were employed. The duration of direct voltage application ranged from 0·01 to 300 sec. A relay circuit was employed to control the applications of short duration.

Alternating voltages of 10 and 20 V peak amplitude in the frequency range 0·1–10 c/s were supplied by a stable, low-frequency resistance–capacitance oscillator

while a step-down transformer acting from the mains supply provided alternating voltages of frequency 50 c/s. Alternating voltages were applied for durations appropriate to provide numbers of cycles from 1 to 3000.

In order to assess the importance of the proximity of the bath electrode to the plant root and to determine which part of the root was most affected by the passage of current during electric stimulation, experiments were conducted to determine the distribution of applied current density flowing out from the plant root surface and into the bathing solution. This was done by exploring the region around the root with a measuring probe to determine the equipotential pattern. From this the current paths were determined and the current strengths passing out from various regions along the root were calculated.

It was found that the current density passing through the region of the root just below the bathing solution surface was about 100 times as great as that passing through the tip region, the current density falling off exponentially between the two regions. Further, the distribution of the applied current passing out from the root was found to be independent of the position of the bath electrode provided that it was situated more than 2 cm below the root tip. In all experiments reported in this paper the separation between the root tip and the bath electrode was greater than this distance. The bathing solution could then be regarded as constituting the bath electrode.

General observations of growth rate and also microscopic examination of plant root sections revealed no injurious effects which might have been caused by the accumulation of ions during the passage of current within the range of currents and durations employed.

In some experiments the rate of elongation of the root was recorded using the growth meter described by Scott (1957). No significant changes were observed in the rate of elongation either during or following electric stimulation.

### III. Results

#### (a) General Form of Transient Potentials

Following stimulation, the plant's potential pattern recovers eventually to a state not significantly different from that preceding stimulation, provided the stimulus is not too great. The form of the transient potential pattern depends on the particular type of stimulus applied and is independent of any previous electric stimuli which the plant has undergone provided sufficient time has been allowed for the potential pattern to recover to the normal unstimulated state.

Figure 1 shows typical time courses of potentials near two representative points along the same plant root for three different successive electric stimuli. Figures 1(a) and 1(b) refer to direct voltage applications of opposite polarity while Figure 1(c) refers to alternating voltage application. Values of potentials prior to stimulation are shown at $t = 0$. From this example it may be seen that the potential transients frequently overshoot the steady value before recovering. In some cases slow damped oscillations of periods up to 40 min have been observed. The general

form of the transients suggests that the potential recovery is of a damped oscillatory nature.
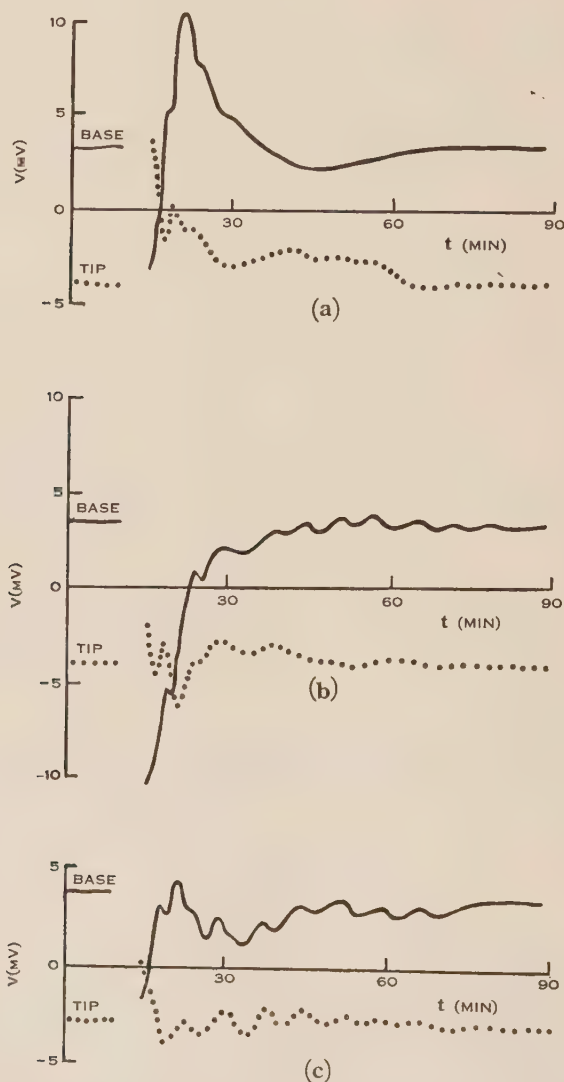


Fig. 1.—Time courses of transient potentials observed at the tip and basal regions of the same root. The stimuli applied are $(a)+10$ V, $(b)-10$ V, and $(c)$ 10 V peak alternating voltage, each applied for 300 sec. Steady potentials before stimulation are shown in each case, the gap in the record indicating the time of stimulation. For simplicity only two of the five transients normally recorded are shown. The three intermediate transients show a gradual transition in form between the two extremes shown.

As well as these long period oscillations, less heavily damped oscillations of shorter period (approx. 5 min) are frequently observed superimposed on the slowly

varying recovery. These shorter period oscillations are similar to the spontaneous oscillations occasionally produced by bean roots (Scott 1957).

In Figures 1(a) and 1(b) it is apparent that the transients following the application of +10 V for 300 sec is different from those following −10 V for the same duration. It is shown in Section III(c) that the direct voltage transients may be regarded as containing a component which is not dependent on the polarity of the applied voltage and a component which is.

Since the transients resulting from alternating voltage stimulation are simpler in form than those for direct voltages, the results of the former will be described first.
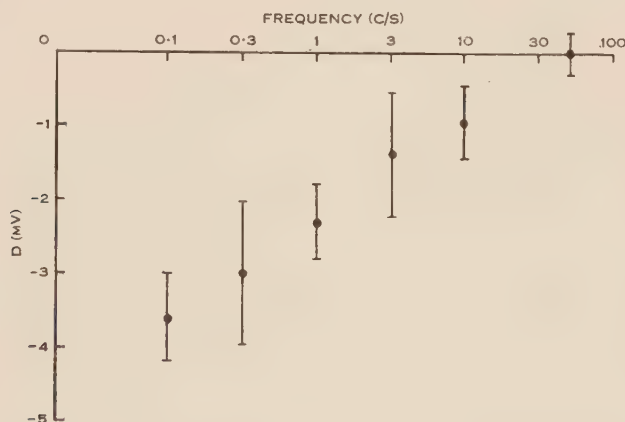


Fig. 2.—Relations between $D$ (at the root base) and the frequency of the 10 V peak voltage applied for 300 sec. Ninety-five per cent. confidence limits are indicated (10–20 plants).

### (b) Alternating Voltage Stimulation

In these experiments it was necessary to ascertain first that the resulting transient was not dependent on the phase in the cycle at which the alternating voltage stimulus was switched off. It might be reasonable to expect, for instance, that after applying one cycle of relatively long period, such as 10 sec, that the recovery transient would differ if the cycle were applied between two positive peaks (maxima), or between two negative peaks (minima). This was tested by averaging the appropriate transients obtained from 10 plants, each of which received a number of stimuli of the two extreme types cited. It was found that the corresponding pairs of average potential recovery curves did not differ significantly from one another. Hence it was concluded that transients caused by alternating voltage application are not dependent on the phase of the alternating cycle (or cycles) applied.

As shown in Figure 1(c), the characteristic feature of the alternating voltage transients is that the root tip potentials are algebraically increased while those of the root base are decreased immediately after stimulation, i.e. the electric polarity of the root tends to reverse. In general, the potential is decreased only at the root

base, i.e. in the near vicinity of the bathing solution surface where most of the
applied current flows through the root surface, the rest of the potentials being
increased, though often to a lesser extent.
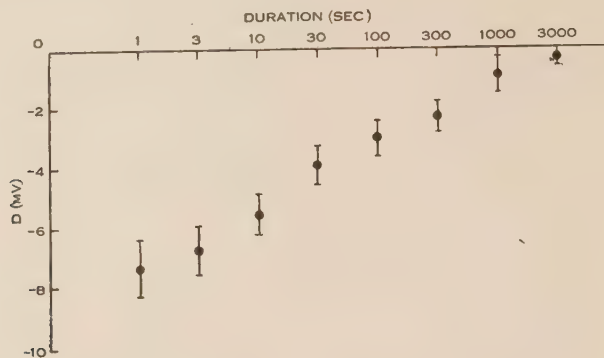


Fig. 3.—Relation between $D$ (at the root base) and the
duration of the applied 10-V stimulus at 1 c/s. Ninety-five
per cent. confidence limits are indicated (20 plants).

It is evident that an index of the effect caused by stimulation is given by the
deviation in plant potential (at a particular point along the root) immediately after
removal of the stimulus. This deviation ($D$) with sign attached, may then be used
to compare the effects caused by different electric stimuli.
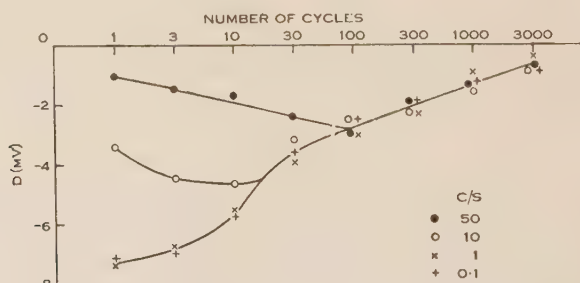


Fig. 4.—Relation between $D$ (at the root base) and the
number of cycles of the applied 10 V peak voltage at
frequencies of 50, 10, 1, and 0·1 c/s. Ninety-five per cent.
confidence limits are similar in magnitude to those in
Figure 3 (20 plants).

In Figure 2, $D$ (at the root base) is plotted against the frequency of the
alternating voltage applied for a constant duration of 300 sec, while, in Figure 3,
$D$ is plotted against duration of application at a constant frequency of 1 c/s. In
both cases the peak voltage was 10 V throughout.

It is seen that the magnitude of $D$ decreases with both increasing frequency
and duration of application. This suggests that the magnitude of $D$ decreases with
the number of cycles of alternating voltage applied. In Figure 4, $D$ is plotted against

the number of cycles, employing frequencies of 50, 10, 1, and 0·1 c/s, applied for appropriate durations.

It appears that $D$ depends uniquely on the number of cycles applied at a constant peak voltage, except for the cases in which the stimulus is applied for durations less than about 1 sec (viz. 50 c/s for 1–30 cycles, and 10 c/s for 1–10 cycles).

The fact that for very brief applications of the higher frequency alternating voltages, the curve departs from the unique relation between $D$ and the number of cycles, implies that insufficient electricity is passed through the plant during such short applications. It is possible to pass this required amount of electricity through the plant by applying a single initial cycle of 1 sec period. If this is done and then
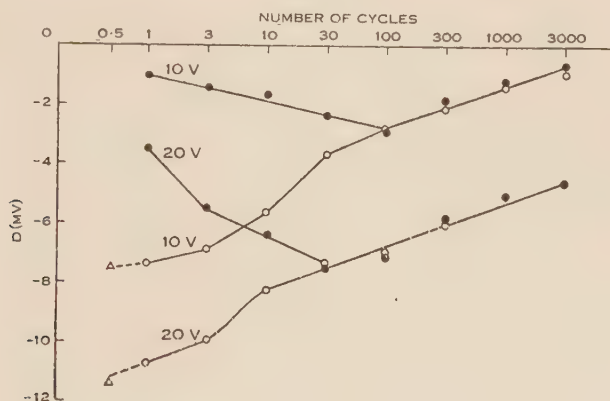


Fig. 5.—Relations between $D$ (at the root base) and the number of cycles at 50 c/s (●) of 10 V (upper curve) and 20 V (lower curve). ○ Values of $D$ obtained when a single cycle of period 1 sec is applied immediately before the 50 c/s voltage application. Ninety-five per cent. confidence limits for all points are similar to those in Figure 3 (15 plants). Points at 0·5 cycles (△) are referred to in Section IV.

followed immediately by a number of cycles of frequency 50 c/s, the relation between $D$ and the number of cycles then becomes identical with that for lower frequencies such as 1 and 0·1 c/s (Fig. 4) even if the number of cycles is small.

This is shown in Figure 5 in which the 10 V 50 c/s curve from Figure 4 is redrawn. The other 10 V curve (open circles) shows 10 V 50 c/s data obtained after first applying an initial cycle of 1 sec period. It is apparent that this curve is identical with that in Figure 4 for the 1 c/s and 0·1 c/s points.

Data obtained similarly for 20 V 50 c/s is shown in Figure 5 also. The relations between $D$ and the number of cycles at 20 V are obviously similar to those at 10 V but the magnitude of $D$ is greater. The point at which the upper and lower constant voltage curves join indicates the number of cycles for which just sufficient charge has been passed through the plant to make the relation between $D$ and the number of cycles independent of frequency. For 50 c/s at 10 V this point is at 100 cycles, while for 20 V, only 30 cycles is sufficient.

This suggests that the passage of a constant quantity of charge is necessary before the dependence of $D$ on the number of cycles becomes independent of the

frequency. As is shown below, it appears that this required quantity of charge is independent of the applied voltage and its frequency.

Values of this quantity of charge $Q$ have been determined from measurements of the average value of alternating current passed through plants during stimulation. $Q$ is then given by the product of the average current and the necessary duration for the dependence of $D$ on the number of cycles to become independent of frequency. Three cases have yielded the following average values of $Q$ (Table 1). Twenty plants were used in each case. Considering the logarithmic type of relation between $D$ and the number of cycles, there is quite good agreement between the values of $Q$ obtained.

TABLE 1

INDEPENDENCE OF QUANTITY OF CHARGE ON APPLIED

VOLTAGE AND FREQUENCY

Each result is the mean of 20 plants

| Voltage (V) | Frequency (c/s) | $Q \times 10^5$ (C) |
|---|---|---|
| 10 | 10 | $9 \pm 5$ |
| 10 | 50 | $14 \pm 5$ |
| 20 | 50 | $10 \pm 5$ |

The form of the relations between $D$ and the number of cycles at other points along the root is identical with those described for the basal regions (i.e. at points on the root just below the surface of the bathing solution). However, as mentioned previously in this section, $D$ changes sign and decreases in magnitude a few millimetres below the bathing solution surface.

### (c) Direct Voltage Stimulation

From sets of transients such as those of Figures 1(a) and 1(b) obtained from a number of plants, it is found that the average recovery curves of potential are significantly different depending on whether the direct voltage is applied in a positive or in a negative sense with respect to the bathing solution. However, the averages of corresponding pairs of transients induced by equal but opposite direct voltage stimuli in a number of plants are found to be very similar to those obtained from alternating voltage stimulation. These average curves will be referred to as the "common" components of the direct voltage transients and may be regarded as their components not dependent on the sign of the applied voltage. Only the common components will be discussed in this paper. As in the alternating voltage results, the characteristic feature of the common components is that the potentials of the very basal end of the root are decreased while those of the regions nearer the tip are algebraically increased immediately after removal of the applied direct voltage. Again, an index of the common effect may be defined as the initial deviation,

$D_c$, following stimulation of the common potential curve from the unstimulated plant's potential.

Figure 6 shows $D_c$ (at the base) plotted against duration of application of both 9 and 18 V. These graphs show that the magnitude of $D_c$ increases with duration up to about 1 sec for 9 V and about 0·3 sec for 18 V after which $D_c$ is independent of the duration of voltage application but its magnitude increases with voltage.
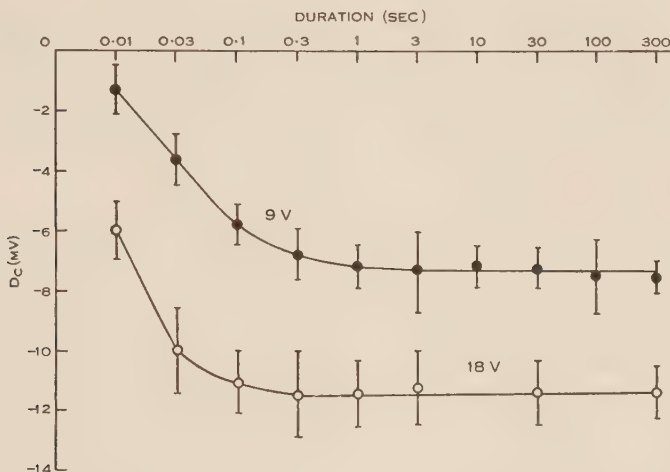


Fig. 6.—Relations between $D_c$ (at the root base) and the duration of application of 9 and 18 V. Ninety-five per cent. confidence limits are indicated (20 plants).

This again indicates a charge requirement before $D_c$ becomes independent of duration, the average value of this charge $Q_c$ for the two cases (9 and 18 V) being $9 \times 10^{-5}$ C. This may be compared with the average value of $Q = 11 \times 10^{-5}$ C, obtained in the alternating voltage case (Section III($b$)).

## IV. Discussion

In Section III($b$) it was seen that $D$ depends uniquely on the number of cycles of constant peak voltage applied, provided that the amount of charge passed through the plant during stimulation exceeds a definite quantity $Q$ ($= 11 \times 10^{-5}$ C approximately). This quantity of charge would be transported through the plant by $11·4 \times 10^{-10}$ g-equiv. of ions. It was shown further (Fig. 5) that after this amount of charge has been passed by applying a single cycle of period 1 sec, the relation between $D$ and the number of cycles at 50 c/s applied subsequently is identical with the unique relation between $D$ and the number of cycles applied at lower frequencies such as 1 and 0·1 c/s, at which frequencies the required amount of change is passed during only one cycle.

The relation between $D_c$ and duration of 9- and 18-V pulses was shown to exhibit a similar effect in that the magnitude of $D_c$ increases with duration until

a charge of $Q_c$ ($= 9 \times 10^{-5}$ C) has been passed, after which $D_c$ remains constant (Fig. 6).

A single direct voltage pulse of about 10-V amplitude might be interpreted as half a cycle of 10-V peak alternating voltage. In this case it would be expected that $D_c$ should be independent of the pulse length (provided it is 1 sec or longer) since the number of cycles (viz. a half cycle) remains the same whatever the pulse length is. This may be compared with the alternating voltage case in which single cycles of period 1 sec or longer were seen to cause identical values of $D$.

Figure 5 shows the 10- and 20-V curves relating $D$ at the base and the number of cycles. The points shown at 0·5 cycle indicate the values of $D_c$ for 9- and 18-V pulses of duration greater than 1 sec. It is apparent that these points are close to the extrapolations (dotted) of the 10- and 20-V alternating voltage curves respectively.

This, together with the fact that the values of $Q$ ($= 11 \times 10^{-5}$ C) and $Q_c$ ($= 9 \times 10^{-5}$ C) are in agreement, suggests that the direct voltage common effect and the alternating voltage effect are identical.

Having combined the two effects in this way, the phenomenon may be summarized as follows. The transient potentials observed immediately after the removal of an electric stimulus depend on two factors: the total quantity of charge passed through the plant, and the number of alternations of the applied voltage. As the quantity of charge passed through the plant is increased the magnitude of the transients (measured by $D$ or $D_c$) increases until its maximum value is reached with the passage of approximately $10^{-4}$ C. This quantity of charge and the value of $D$ or $D_c$ is independent of the manner in which the charge is passed although the value of $D$ or $D_c$ increases with the magnitude of the applied voltage.

In addition to this effect there is that of the number of cycles in which the magnitude of $D$ decreases approximately logarithmically with the number of cycles applied.

Marsh (1930) showed that following the passage of direct current along onion (*Allium cepa*) roots, the potential changed and eventually returned to the un-stimulated state. The potential change was found to increase with the amount of charge passed along the root while the polarity of the potential change depended solely on the direction in which the current flowed, i.e. no common change was observed even though the quantities of charge passed through the roots ranged from $10^{-5}$ C to values as large as $10^{-3}$ C. However, these experiments were conducted in a rather different manner from those described in this paper. The electrodes, used both for measuring root potentials and for the application of electric current, consisted of ring electrodes containing tap water placed at various positions along the root growing in air saturated with water vapour.

It is to be expected that both the stimulated and the unstimulated root's potential pattern should differ for the two methods of measurement, since potentials, either steady or transient, produced in a bathing solution depend on the flow of bioelectric current through the solution about the root (Scott, McAulay, and Jeyes 1955), whereas for a root in air, a more static potential is measured.

Danisch (1921) showed that *Vorticella nebulifera* adapts itself to the application of a succession of mechanical shocks of known energy. For instance, after nine impulses, each of 500 ergs, the organism no longer responds to repeated impulses, whereas for 2000-erg impulses, 420 are required. Danisch concluded that the effect was one of habituation rather than fatigue. These results appear to be similar to those described in this paper.

## V. Acknowledgments

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# NODULATION STUDIES IN LEGUMES

## II. THE INFLUENCE OF VARIOUS ENVIRONMENTAL FACTORS ON SYMBIOTIC EXPRESSION IN THE VETCH (VICIA SATIVA L.) AND OTHER LEGUMES

### By J. S. Pate*

### Summary

The major sequences of symbiosis in field-grown vetch plants (*Vicia sativa* L.) are similar to those described in Part I of this series (Pate 1958) for the field pea (*Pisum arvense* L.). In both species symbiotic development is found to be characteristically synchronized with host plant leaf production and nitrogen accumulation. A generalized scheme for the symbiosis and nitrogen economy of the annual legume is derived from nodulation studies carried out in Australia and Northern Ireland.

Symbiotic expression of vetch as a winter annual is essentially similar to that of the same plant grown as a summer annual. There is no evidence of impairment of nodule growth and efficiency, or curtailment of nodule span in vetch plants overwintering in Northern Ireland.

Plant flowering induces marked decreases in nodule number and total nodule weight in the annual legume. Nodule losses in vetch may be temporarily arrested by regular removal of flower buds. Continued root growth on plants with artificially extended vegetative phase permits development of a larger nodule complement on minor roots than on similarly aged flowering plants. The significance of this "flowering factor" in annual legume symbiosis is discussed.

Qualitative studies of the seasonal nodulation of various Irish legumes depict successful overwintering of nodules as a common event in all winter annual, biennial, and perennial species. Biennial nodules are described for the woody perennial genera *Ulex* and *Sarothamnus*. The nodulation sequences of all legume species studied are found to be determined largely by changes associated with general host plant development, e.g. periods of root expansion and rapid plant growth, the onset and completion of reproductive phases of the life cycle, etc. Examples are described where climatic and biotic effects are superimposed on this inherent symbiotic pattern of the legume.

## I. Introduction

Remarkably little information exists on the normal life span and turnover of nodules on wild or cultivated legumes in field conditions. Even less is known of the effect and action of host plant and environment factors on nodule functional life.

It is generally agreed that in temperate climates the normal life of an effective nodule is for only one season or growing period. According to Russell (1952) biennial and perennial herbaceous legumes generally conform to the plan of an annual renewal of nodules. Jones (1943) points out that nodules on tap-rooted legumes are frequently developed on annual investments of non-cambial roots and hence are unlikely to

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survive into a second season. For annual species, Fred, Baldwin, and McCoy (1932) describe host-induced nodule shedding in reproductive stages of the plant life cycle. They state that a sudden disappearance of nodules occurs in flowering and fruiting of large-seeded legumes, while nodule shedding extends over a longer period in the host plant cycle of small-seeded species.

Certain tropical tree and shrub legumes are exceptional in possessing perennial nodules which exhibit continued growth activity in the form of annual or seasonal increments of bacterial tissue. Such lobed nodules have been described for the genera *Sophora* and *Acacia* (Spratt 1919), *Wistaria* (Jimbo 1927), *Sesbania* (Harris, Allen, and Allen 1949), and *Caragana* (Allen, Gregory, and Allen 1955). The above authors associate various anatomical features of the perennial nodule with its prolonged symbiotic activity. Viermann (1929) provides an interesting example of perennial nodules in the herbaceous legume *Lupinus*. In perennial species grown in Germany nodule meristems were shown to be preserved from year to year to give peripheral additions of bacterial tissue during each growing season.

Several environmental factors have been described as influencing nodule longevity. In each case physiological shocks to the host plant are reflected by adverse effects on nodule populations. Drought-induced shedding of large portions of nodule populations has been noted by Wilson (1942). Under Wisconsin climatic conditions he found that several cycles of alternate shedding and renewal of nodules on field-grown legumes followed the onset and alleviation of soil moisture stresses during the growing season. Extensive damage to nodules by larvae of the weevil *Sitonia lineatus* has been described by Mulder (1948) and Masefield (1952) for European legumes. Clipping of leaves (Eaton 1931; Wilson 1931) or shading (Thornton 1930; Strong and Trumble 1939) are stated to induce premature nodule losses, the latter generally attributed to carbohydrate deficiencies in host tissues. It is surprising to find that there is no detailed information available on nodule destruction in relation to the periodic grazing activity of animals.

The purpose of the present paper is to present quantitative observations on climatic, biotic, and host plant influences on nodule longevity in field-grown legumes. Special study is made of nodule survival in overwintering legumes and of the host plant flowering factor in relation to nodule emptying. The quantitative studies on *Vicia sativa* L. also provide an example of synchronized host and symbiotic development remarkably similar to that described for *Pisum arvense* L. in Part I of this series (Pate 1958). An interesting sideline to the *V. sativa* studies is found in the account of effective and ineffective strain behaviour in multiple infection of host plants in field conditions.

## II. Materials and Methods

### (a) Plant Material

Growth and nodulation of vetch (*Vicia sativa* L.) was studied by periodic sampling from three series of field-grown material:

*Series* 1.—Autumn sowing; growing period September 1955–July 1956.
*Series* 2.—Spring sowing; growing period April–July, 1956.
*Series* 3.—Summer sowing; growing period June–October, 1956.

Series 1 and 2 sowings were made in the same soil environment to compare symbiotic performance of vetch as a winter or summer annual. Series 3 sowing was designed to examine the effect of plant flowering on nodule initiation and longevity. It comprised control plants which were allowed to reproduce normally, and a treated series where all flower buds were removed to maintain plants in an artificially prolonged vegetative condition through the declining season.

For each sowing some 600–800 sq. ft of ground were cultivated, plots being chosen from nitrogen-deficient soil of sandy character at Belfast, Northern Ireland. Series 1 and 2 constituted one randomized block experiment. Series 3 had a similar randomization of treated and untreated portions of the plot. Adequate nitrogen-free mineral fertilizer was applied to all plots before the sowings. In the over-wintering series cloche protection was provided from December to April. Seeds of series 2 and 3 were sown 1–1½ in. deep; a deep (3–4 in.) sowing was employed in series 1 to favour autumnal growth and winter hardiness.

None of the sowings was artificially inoculated. In each series multiple infection by indigenous rhizobia resulted in the production of two contrasting nodule types. These proved of ineffective and effective character as gauged by their haemo-globin-pigmented life, nodule meristem activity, and nitrogen fixation potential in pure culture with vetch as host plant (see Section III).

### (b) Sampling Procedure

Sampling methods were similar to those used for field-grown material of *P. arvense* (see Pate 1958), and reference should be made to this paper for details of technique and the relevance and accuracy of the various characters recorded.

A 50-plant sample unit was selected from the plots at 3–7 day intervals in host plant life cycles. Sampling involved a 0·5–3 per cent. standard error in recorded host symbiotic quantities.

### (c) Recordings from Samples

The following plant and nodule characters were recorded for each 50-plant sample in the series:

(i) *Series 1, 2, and 3*

(1) *Leaf Age (expanded leaves/plant).*—As a winter annual, *V. sativa* developed a compact autumn rosette of three to six shoots of limited growth bearing a total of some 10–20 leaves/plant. The following spring, the short shoots died, total leaf number showed a temporary decrease, and then a flowering axis of some 15–25 leaves developed. Similar development occurred in spring or summer sowings but fewer preflowering shoots formed and there was less marked leaf abscission in late vegetative development (see leaf-age scales on Figs. 1–9).

(2) *Nodule Number and Colour.*—Three colour classes were recognized in nodule counts, white (*W*-type) young nodules, red (*R*-type) haemoglobin-pigmented nodules, and green (*G*-type) senescent nodules. Counts were taken of the various nodule colour classes on primary and secondary root systems. The few nodules forming on tertiary roots were classed as secondary root nodules in counts and

weight determinations.   The autumn series showed considerable infection of the
rootlet-invested epicotyl in addition to normal nodulation of primary and lateral
roots. This supracotyledonary infection was undoubtedly related to the deep sowing
of the overwintering series.
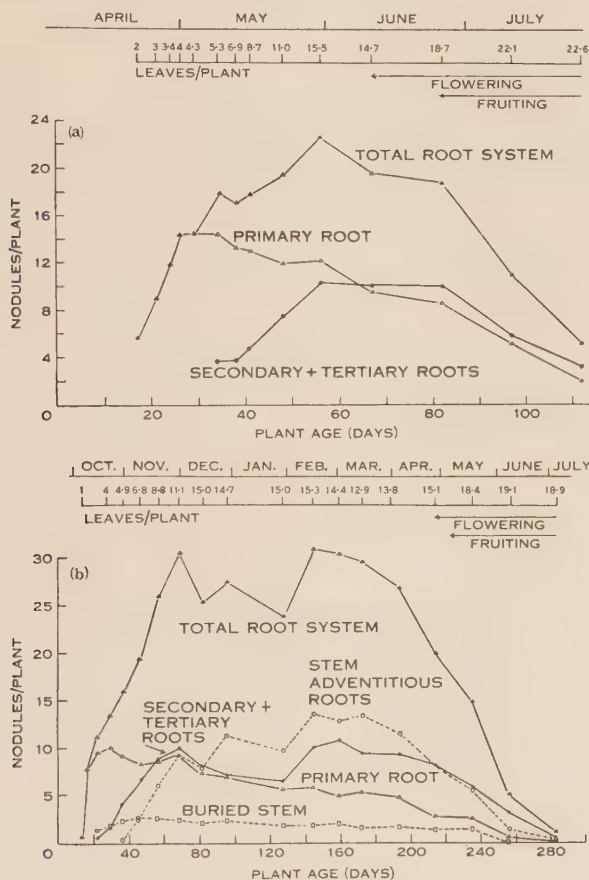


Fig. 1.—*V. sativa* nodulation: nodule numbers on various parts of the
root system. (*a*) As a summer annual (1956 series); (*b*) as a winter annual
(1955–56 series).  Each point on the various curves of Figures 1–9 represents
a mean value derived from a sample of 50 plants.

### (ii) *Additional Recording Series 1 and 2*

Fresh weight determination and nitrogen analyses were made of host plants
organs and nodule colour samples.  Techniques were similar to those used in study
of the nitrogen economy of the field pea (Pate 1958).

### (d) *Qualitative Observations on Nodulation of Native Legumes in Northern Ireland*

Several plant communities containing a total of 29 legume species were kept
under observation over the period 1953–1956.  At monthly intervals sample plants
of the various species were extracted and notes made on rooting characteristics and

size and condition of nodule populations.  Nodule distribution patterns and nodule
longevity cycles were constructed from the data.

## III. Results

### (a) Comparison of Nodulation in Vetch as a Summer and Winter Annual

(i) *Nodule Number* (*Fig.* 1)

     Winter annual development is characterized by autumnal and vernal maxima
in total nodule numbers (Fig. 1(*b*)).  The autumn maximum represents the com-
bined initial infection of all four root systems in the sequence of their expansion—
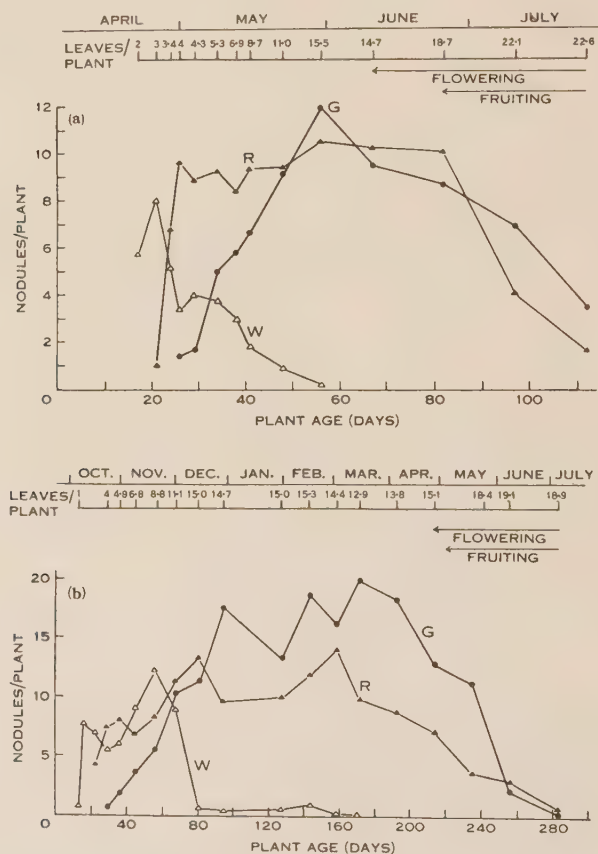


Fig. 2.—*V. sativa* nodulation: nodule colours for complete root system.
*W*, young white nodules; *R*, red actively fixing nodules; *G*, green senescent
nodules.  (*a*) As a summer annual (1956); (*b*) as a winter annual (1955–56).

primary root system, buried epicotyl, secondary roots, epicotyl adventitious roots.
The vernal maximum comprises the additional infection of minor roots following
resumption of root growth in early February.  The spring sowing shows a single
peak in nodule numbers in late vegetative development (Fig. 1(*a*)).

All three vetch series show marked declines in nodule numbers in late vege-
tative development, nodule disappearance being most noticeable on flowering and
fruiting plants.
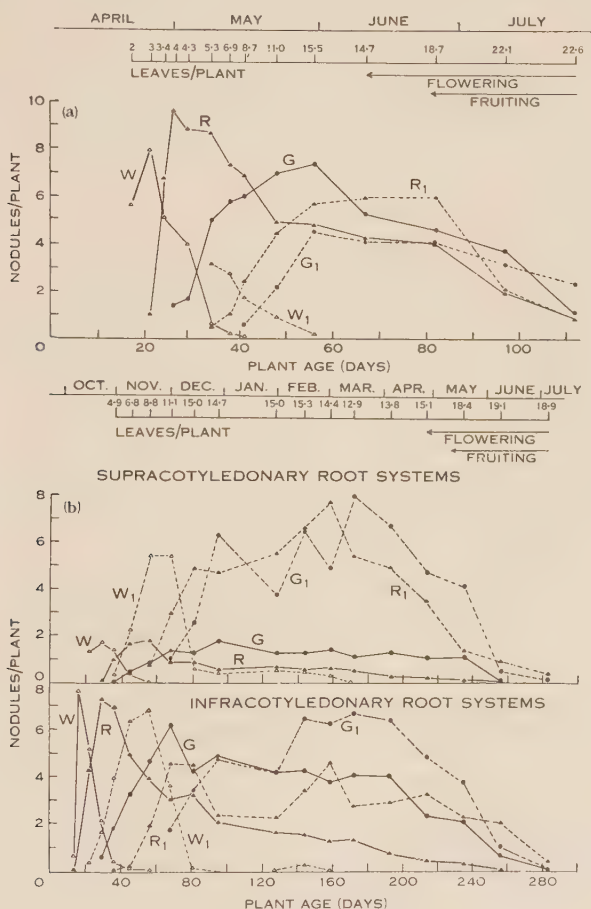


Fig. 3.—*V. sativa* nodulation: nodule colour for various parts of the root system.
*W*, *R*, and *G* symbols as in Figure 2. (*a*) As a summer annual (1956): *W*, *R*, and
*G* curves for primary root; $W_1$, $R_1$, and $G_1$ curves for secondary + tertiary roots.
(*b*) As a winter annual (1955–56): supracotyledonary root systems, *W*, *R*, and *G*
curves for epicotyl nodules and $W_1$, $R_1$, and $G_1$ curves for adventitious roots from
buried epicotyl; infracotyledonary root systems, *W*, *R*, and *G* curves for primary
root and $W_1$, $R_1$, and $G_1$ curves for secondary + tertiary roots.

(ii) *Nodule Colour (Figs. 2 and 3)*

On complete plants or individual root systems of either series, nodule popu-
lations exhibit the same progressive colour changes from white to red to green as
those observed in the life of any effective or ineffective nodule on the roots. This
effect is well illustrated in the winter vetch sowing where successive maxima in
the *W*, *R*, and *G* curves are attained in the same sequence as the four root systems
were expanded in early seedling growth (Fig. 3(*b*)).

(1) *Root Infection* (W *Curves*).—W curves for complete root systems are bimodal, the two peaks corresponding to periods of nodule formation and accumulation of young nodules on primary and secondary roots (Fig. 2(a)). A third minor peak in the overwintering series in February marks nodulation of minor root systems
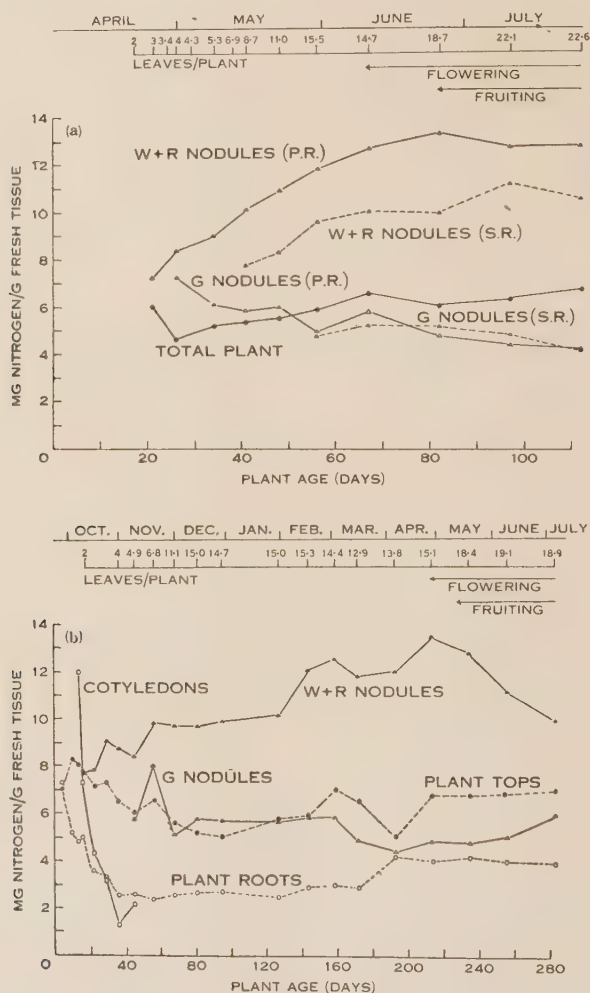


Fig. 4.—*V. sativa* nodulation: nitrogen concentration in nodules and host plant tissues. *P.R.*, primary root nodules; *S.R.*, secondary + tertiary root nodules; *W*, *R*, and *G* symbols as in Figure 2. (*a*) As a summer annual (1956); (*b*) as a winter annual (1955–56).

expanded or added to in spring growth (Fig. 2(b)). Root development and nodule initiation is completed in both series well before flowering commences.

(2) *Nodule Activity and Senescence in Effective and Ineffective Nodules* (R *and* G *curves, Figs.* 2(b), 3(b)).—Nodule pigmentation rates may be determined by comparing ascending portions of the *W* and *R* curves of the various root systems. In *spring* and *summer* sowings, pigmentation of the first few nodules on a root

system took place some 4–8 days after their appearance on the primary root. As in the summer sowings of field pea (Pate 1958), later-formed nodules on root systems were pigmented more quickly (3–5 days) then their earlier-formed neighbours (5–9 days). In the *winter* sowing, adverse effects of decreasing light and temperature on host plant growth produced an opposite effect to the above. Thus, nodules on the primary axis were pigmented in 7–19 days in October, while those which developed 6 weeks later on lateral root systems remained white for 18–30 days before acquiring visible pink coloration. It may be concluded that haemoglobin production is closely related to host plant growth rate as influenced by current climatic conditions.

The two series showed invasion by native soil rhizobia of two major and contrasting strain types giving highly effective and highly ineffective symbiosis with *V. sativa*. Approximately equal proportions of the two nodule types appeared on the roots of the series. The following observations were made on the biology of the two strain reactions both in the conditions of the experiment and in sterile cultures of vetch in association with the two classes of rhizobial variants:

*Ineffective Nodules.*—These were small, hemispherical structures with meagre and transient meristematic activity and an active (red) life of 5–8 days in summer conditions. Mature fresh weight of the ineffective nodule was about 0·5–1·0 mg, less than 5 per cent. of the size of mature effective nodules. Anatomical investigations revealed an early disorganization of the nodule meristem as a primary cause of ineffectiveness. Less than 10 per cent. of the bacterial tissue of the nodule was comprised of bacteroid-filled cells, despite the presence of numerous infection threads. The latter remained filled with rod-shaped bacteria for periods of up to 10 weeks after haemoglobin destruction had been completed in the nodule.

Once-green ineffective nodules persisted on root systems for long periods, many remaining intact until root decay on fruiting plants. It is not known why these ineffective nodules were so highly resistant to the internal and external decay agencies which quickly disintegrated green effective nodules on vetch and other legumes in field conditions.

Sterile cultures of vetch with the ineffective *Rhizobium* gave no evidence of nitrogen fixation activity when compared with nitrogen-free uninoculated controls. Indeed, in plants exhibiting multiple infection by effective and ineffective rhizobia, a load of green, persistent nodules might deplete host carbohydrate reserves and thereby detract from the full benefit of effective nodule activity. Study is now being made of this latter aspect of symbiosis.

The progressive accumulation of ineffective members of nodule populations accounts for the very high numbers of green nodules in many samples of the three series (cf. the rapid elimination of senescent members of the effective nodule populations of the field pea series described in Part I).

*Effective Nodules.*—These were large cylindrical structures with a mature average weight of 20–30 mg. They showed an active life of from 20 days to the death of host plants (i.e. as long as 200 days in overwintering primary root nodules). More than 90 per cent. of nodule bacterial tissues was comprised of swollen, bacteroid-filled cells.

In aging effective nodules, haemoglobin destruction commenced in proximal tissues and proceeded towards the nodule meristem. Completely green nodules persisted for only a few days on root systems before decaying and being sloughed off into the surrounding soil.
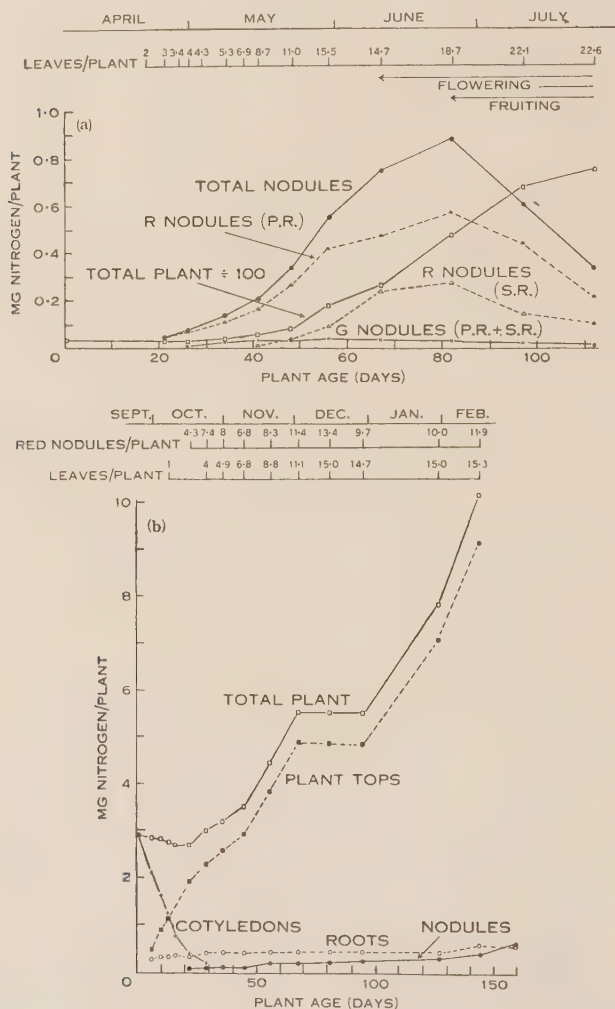


Fig. 5.—*V. sativa* nodulation: nitrogen content of nodule and host plant tissues. (*a*) As a summer annual (1956); (*b*) seedling nitrogen economy in the overwintering series (1955–56).

### (iii) *Nitrogen Contents of Plant Tissues (Figs. 4–6)*

Many of the salient features of the nitrogen economy of the two vetch sowings are similar to those described for field pea nodulation. To avoid undue repetition only the more important results summarized in Figures 4 and 5 will be mentioned here.

(1) *Nitrogen Accumulation in Nodules*.—On a fresh weight basis, nitrogen levels in red nodules rise as nodule populations age. Nitrogen concentrations in
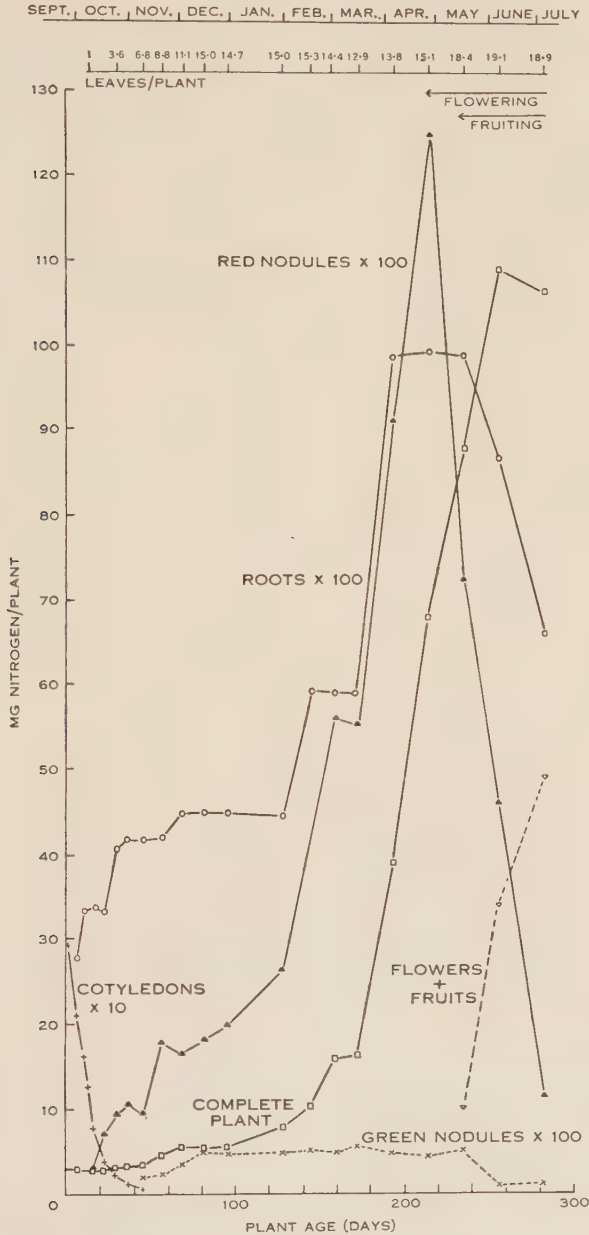


Fig. 6.—*V. sativa* nodulation: nitrogen content of host plant and nodule tissues over complete life cycle of the winter annual series (1955–56).

nodules on minor roots (series 2, Fig. 4(*a*)) are consistently lower than in nodules on the main root axis. At all stages of plant growth, *G* nodule nitrogen levels are

some 40-60 per cent. of those in $R$ nodule samples of similar age. Total red-nodule nitrogen increases to a maximum in early flowering, some time before a maximum in plant nitrogen is established (Figs. 5($a$) and 6).

In Part I it was suggested that the red-nodule nitrogen content of the plant might serve as a useful approximation to the current fixation potential of the symbiotic organs. The potential fixation activities of secondary root, and primary root nodule populations over the life cycle of series 2 may be compared with reference to the areas under the red-nodule nitrogen curves for these root systems (Fig. 5($a$)). Such a comparison would appropriate a possible fixation contribution from primary
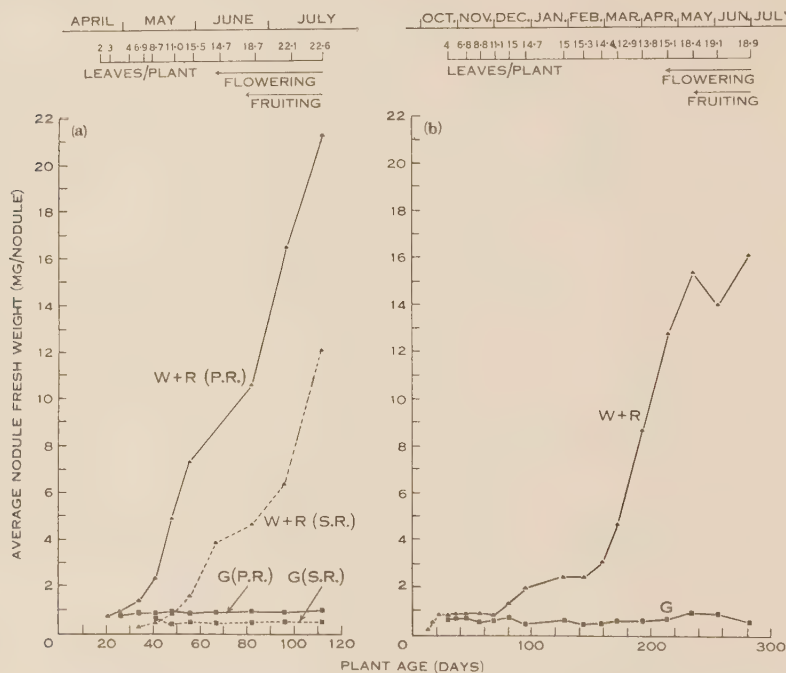


Fig. 7.—*V. sativa* nodulation: average nodule fresh weight (mg/nodule). (*a*) As a summer annual (1956); (*b*) as a winter annual (1955–56); $W + R$, white + red nodule sample; $G$, green nodule sample; $P.R.$, primary root; $S.R.$, secondary + tertiary roots.

roots some three times greater than that of secondary roots. However, there is no evidence of equal fixation efficiency in secondary and primary root red nodules on a weight or nitrogen basis. Experiments are being designed to cover this aspect of symbiosis.

(2) *Nitrogen Accumulation in Host Tissues.*—Seedling nitrogen nutrition is outlined for the overwintering series in Figure 5(*b*). Total plant nitrogen commences to rise in 25-day-old seedlings as nodules colour and commence fixation. Root nitrogen remains fairly constant over the 30–150-day period, although nitrogen in aerial portions is increased five times during this period. In later stages of growth nitrogen is again preferentially sequestered by the shoot system until at plant

maturity more than 99 per cent. of total plant nitrogen is concentrated in plant tops (Fig. 6). Root nitrogen concentrations are consistently lower than in other plant organs (Fig. 4(b)).

There is thus abundant evidence of an immediate transfer of fixation products to plant tops at all stages of vetch development, with neither roots nor nodules functioning as nitrogen storage organs.
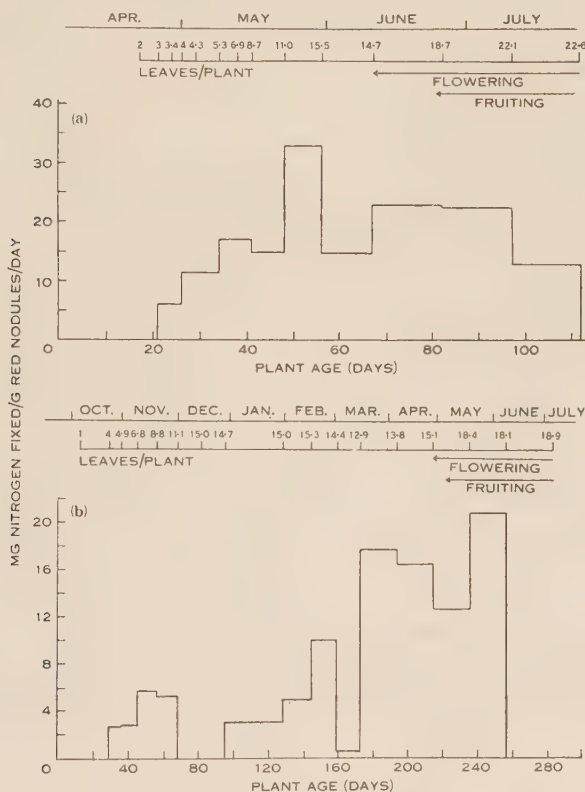


Fig. 8.—*V. sativa* nodulation: fluctuations in apparent nodule fixation efficiency. (*a*) As a summer annual (1956); (*b*) as a winter annual (1955–56).

Both series show sigmoid curves for increase in total plant nitrogen with time. Nitrogen redistribution to flowering parts in series 1 occurs largely at the expense of nitrogen stored in plant tops (Fig. 6). The general nitrogen economy patterns described here for vetch and field pea are essentially similar to those described for non-legume annual species by Williams (1955).

(iv) *Average Nodule Weight* (*Fig.* 7)

W + R *Nodules.*—Average nodule size (fresh weight) in both series increases from less than 0·5 mg to 15–20 mg. Nodules continued to grow through the winter period in series 1 with a sudden increase in growth rate following resumption of plant growth in March. Average nodule sizes of primary and secondary root nodules

are recorded in series 2 (Fig. 7(*a*)). Sigmoid growth curves are depicted, with nodules consistently smaller on the finer lateral roots.

G *Nodules.*—It has been mentioned already that *G* samples in both series were largely composed of persisting ineffective nodules. Hence the average weight of *G* nodules approximates to the mature size of an ineffective nodule and, since the latter does not continue to grow once green, average size remains fairly constant over the life cycle at a value just less than 1 mg/nodule. In series 2 (Fig. 7(*a*)) *G* nodules on secondary and tertiary roots are seen to be consistently smaller than on the primary root.

### (v) *Apparent Fixation Intensity (Fig. 8)*

The histograms of Figure 8 depict fluctuations in the apparent efficiency of red nodules over the life cycles of the two series. Results are expressed as milligrams nitrogen fixed/gram fresh weight of red nodules/day, assuming that all nitrogen accumulated above cotyledon level is derived from nodule fixation. This is considered a reasonable approximation for the extreme conditions of nitrogen deficiency encountered in the plots. In both series it can be seen that the tempo of nodule activity increases as plant flowering is approached (cf. similar results for *P. arvense* (Pate 1958)). In the overwintering series, fixation ceased temporarily in cold weather in December and early January. A second apparent depression of nodule activity over the 160–170-day period coincided with a period of leaf loss following "scorching" under cloches in early spring.

### (b) *The Effect of Flower Bud Removal on Nodulation of Vetch as a Summer Annual (Series 3)*

Fluctuations in nodule numbers and colours are recorded in the three graphs of Figure 9. The vertical line through the figure marks the point where flowering commenced and where flowers were first removed in the treated series. The leaf-age scales for the series show greater leaf production in the treated series.

### (i) *Nodule Number (Fig. 9(a))*

Twenty-five days after flower removal commenced, plants of artificially extended vegetative phase showed significantly greater nodule numbers than control flowering plants. Three weeks later more than three times as many nodules remained on secondary roots of plants of the treated series.

### (ii) *Nodule Colour*

The graphs in Figures 9(*b*) and 9(*c*) express nodule colour classes as percentages of the total nodules present on primary and secondary+tertiary roots respectively. No significant differences are evident in colour or number changes in the primary root system indicating that flower removal has no visible effect on the fate of existing nodules on the primary axis.

A significantly higher proportion of red nodules is recorded for secondary roots of plants of the treated series. Also white nodules were seen to form on tertiary roots of this series right to the end of the experiment, indicative of con-

tinued root expansion in the prolonged vegetative phase. By contrast, nodule initiation continued in the control series only until 30 days after flowering.
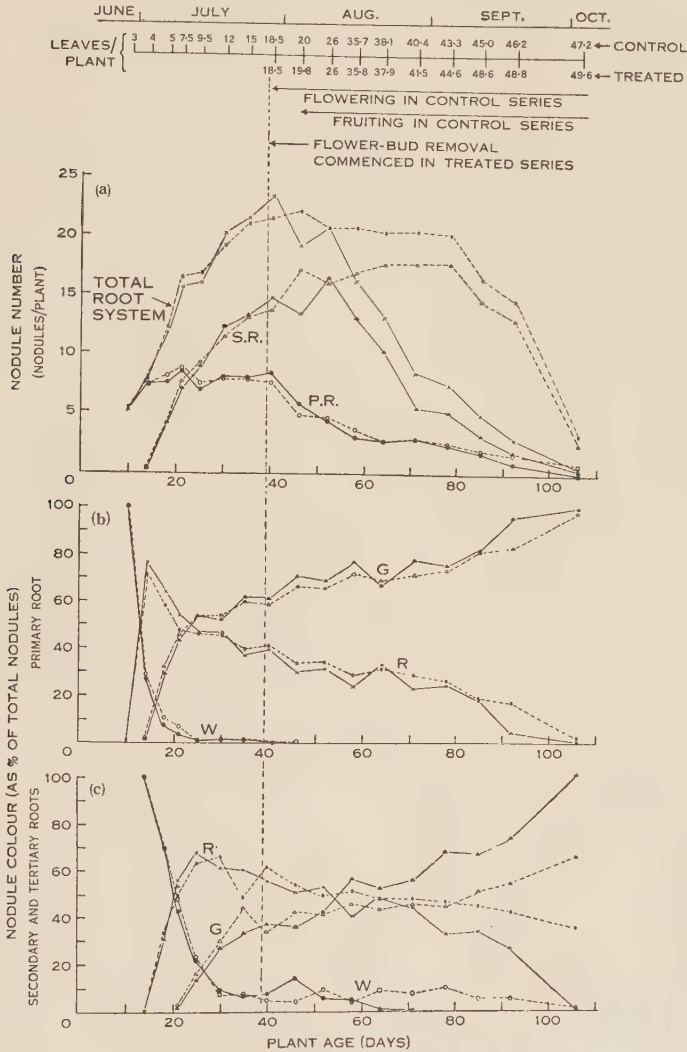


Fig. 9.—*V. sativa* nodulation: the effects of flower-bud removal on nodule production and nodule destruction in a summer sowing of vetch (1954). (*a*) Effects on nodule number. (*b*), (*c*) Effects on nodule colour on primary root, and secondary + tertiary roots respectively. *W*, young white nodules; *R*, red actively fixing nodules; *G*, green senescent nodules; *P.R.*, primary root; *S.R.* secondary + tertiary roots. - - - - Treated series (all flower buds removed). ——— Control flowering series.

The greater number of nodules on minor roots of the treated series may thus be analysed as being due to both a prolongation of nodule initiation on tertiary roots and an increase in the life span of nodules already existing on secondary roots.

### (c) Nodule Longevity Cycles in Native Legumes in Northern Ireland

The 29 species of legumes examined conform to one of six major root growth types. Four distinct nodule longevity cycles are found to operate in normal seasons in Northern Ireland, three of these involving a regular overwintering of nodules. Results of examinations for four seasons are summarized below:

### (i) Rooting Patterns

Type 1: *Fibrous-rooted Annual.*—Characteristic of summer and winter annuals where nodulation is similar to that described for *V. sativa* and *P. arvense*. Nodules, if effective, usually remain on the primary and secondary root until the fruiting of the host plant. Autumnal and vernal root growth, with attendant nodule initiation, was observed in all overwintering species (cf. Section III(b)).` Examples: *Trifolium procumbens* L., *T. dubium* Sibth., *T. arvense* L.; *Medicago arabica* Huds., *M. lupulina* L.; *Vicia hirsuta* Gray, *V. sativa* L., *V. angustifolia* L., *V. lathyroides* L.

Type 2: *Tap-rooted Perennial or Biennial.*—In seedling growth a sparsely nodulated fibrous root system is developed. The primary axis of this system commences to store starch. In subsequent seasons new growth regions of secondary roots are infected, and an investment of nodulated roots is developed annually from latent root primordia on the tap root. Lower portions of extensive root systems may not develop nodules especially where roots are in poorly aerated soil. Examples: *Anthyllis vulneraria* L.; *Melilotus officinalis* Willd.; *Onobrychis sativa* L.; *Trifolium pratense* L., *T. hybridum* L.

Type 3: *Tap-rooted Perennial with Suckers.*—Similar nodulation to type 2, but with a few nodules developed on adventitious roots on the sucker shoots arising from the crown, e.g. *Lotus corniculatus* L., *L. uliginosus* Schkuhr.

Type 4: *Tap-rooted Perennial with Runners.*—Both the original root system and nodal adventitious roots are nodulated. Runners may become isolated from the parent shoot stock and develop into independent, nodulated plants, e.g. *Trifolium repens* L., *T. fragiferum* L.

Type 5: *Woody Perennial.*—The expanding root system is progressively nodulated. In the second and later years of growth older nodules are sloughed off the root system until, in mature plants, nodules are to be found only on minor fibrous roots. In water-logged or poorly aerated soils nodules are restricted to roots exploring the upper soil horizons. Examples: *Sarothamnus scoparius* Koch; *Ulex europaeus* L.; *U. galli* Planch.

Type 6: *Rhizomatous Perennial.*—A short-lived, fibrous system is developed and nodulated in the first few months of seedling growth. This is replaced by an extensive system of starch-storing rhizomes, each with its own adventitious roots. Portions of the rhizome system may become separated from the parent plant. Nodules predominate on adventitious roots from the current season's rhizomes. Examples: *Lathyrus montanus* Bernh., *L. pratensis* L., *L. palustris* L.; *Vicia cracca* L., *V. sylvatica* L., *V. sepium* L.; *Ononis repens* L.; *Trifolium medium* L.

### (ii) Nodule Longevity

Cycle 1: *Summer Annual, Root Pattern Type 1.*—The cycle is essentially similar to that described for summer vetch and field pea, and gives a maximum nodule

life of 6–8 months. White nodules are present only while root systems are expanding; green nodules tend to accumulate in large numbers in the closing stages of the plant life cycle.

*Cycle* 2: *Winter Annual, Root Pattern Type* 1.—Nodulation follows periods of root growth in autumn and spring. Survival of the autumn nodule set varies with the season. For example some 80 per cent. of nodules on *Vicia angustifolia* and *Trifolium dubium* failed to survive the 1953 winter, while in the next two years most nodules overwintered successfully. Maximum nodule life for this cycle is 10–11 months.

*Cycle* 3: *Biennial and Perennial Species, Root Pattern Types* 2–6.—The season's nodule complement is completed in early spring. Nodules may remain on a root system for 12–14 months and hence two sets of nodules may coexist for a short period on root systems in early spring, the older set still haemoglobin-pigmented and presumably active in fixation. It is possible that one aging set of nodules may provide the necessary growth materials for juvenile members on the same root system. A large proportion of each season's nodule set survive the normal Irish winter.

*Cycle* 4: *Woody Perennial Species, Root Pattern Type* 5.—In several gorse (*Ulex europaeus*) and broom (*Sarothamnus scoparius*) populations near Belfast, nodules at least 20 months old were noted on several plants in their third and later years of growth. The life cycle of these "biennial" nodules was further studied by "tagging" individual nodules in pot-grown plants. It was found that nodules which developed on young roots in spring or early summer remained active in fixation until the following autumn when their lower halves turned green. In those few nodules destined to resume growth, the nodule meristem was preserved and allowed a second season's increment of bacterial tissue to be made to the existing bacterial tissue. At the end of its second season the nodule had become an elongate structure, lower half brown and shrivelled, upper half composed of healthy, haemoglobin-pigmented tissue. There was no evidence that any nodules persisted into the spring of a third season, yet this 20-month longevity cycle is reminiscent of the perennial nodules observed on many tropical woody genera.

## IV. DISCUSSION

In Part I (Pate 1958) evidence was given of a characteristic integration of host and symbiotic development in the annual legume *Pisum arvense*. The results presented here describe similar host–*Rhizobium* relationships in further legumes and in addition demonstrate that symbiotic expression is sensitive to biotic and climatic influences. The contributions of the sections of the experimental results are discussed below.

### (a) *Quantitative Studies of* V. sativa *Nodulation as a Summer or Winter Annual*

Figure 10 compares the synchronization patterns for leaf production, plant nitrogen accumulation, and symbiotic development in the winter annual and summer annual sowings. The two series show remarkably similar sequences of attainment of various symbiotic maxima despite marked differences in host plant growth and

final yield. The major differences between the series relate to the climatic effects on symbiosis following exposure to frost and host plant quiescence in the winter annual. The processes of nodule initiation, nodule growth, and nitrogen fixation are clearly interrupted by winter conditions, but there is no evidence of any curtailment of nodule life, or reduction in final nodule size and efficiency in the subsequent development of overwintering nodules.
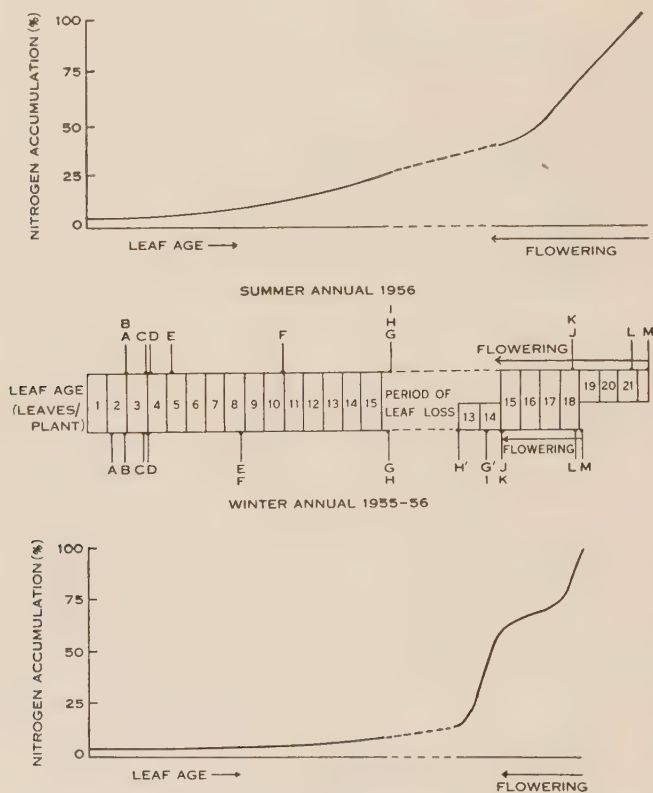


Fig. 10.—Diagram summarizing the synchronization patterns for host and symbiotic development in *V. sativa* grown as a summer or a winter annual. Host plant nitrogen accumulation (expressed as a percentage of total nitrogen present at plant maturity) and various features of the nodulation cycles in the two series are compared on a common leaf-age scale. *A*, maximum primary root white nodules; *B*, first nodule turns red; *C*, maximum primary root red nodules; *D*, 90 per cent. of cotyledon nitrogen exhausted; *E*, maximum secondary roots white nodules; *F*, maximum in nodules/plant fresh weight ratio; *G*, maximum nodules on total root system (*G'*, vernal maximum in overwintering series); *H*, maximum red nodules on total root system (*H'*, vernal maximum in overwintering series); *I*, nodule initiation ceases; *J*, maximum nitrogen concentration in red nodules; *K*, maximum in red nodule nitrogen/plant; *L*, 50 per cent. of red nodules destroyed; *M*, maximum in total plant nitrogen.

Multiple infection in the two series illustrates the rhizobial specificity of symbiotic expression. Annual legume symbiosis involving an ineffective or poorly effective bacterial relationship has the obvious disadvantage of a single setting of nodules which can contribute only an early and meagre fixation return to the host

plant. The ineffective relationship described here for *V. sativa* is peculiar in showing persistent, inactive nodules of life span equal to that of companion effective nodules. Thus the short functional life and limited growth capacity of nodules which characterize this type of ineffectiveness are not accompanied by the early necrosis of nodule tissues specified in many other descriptions of symbiotic maladjustment (e.g. see Thornton 1954 and the recent appraisal of ineffectiveness by Bergersen 1957).

Similar proportions of ineffective and effective nodules developed in the two sowings although different climatic conditions prevailed in the respective periods of infection. It would be interesting to know whether nodulation from mixed soil populations of *Rhizobia* is a property little influenced by environment. Studies are now being made of this aspect of legume symbiosis.

### (b) *Comparisons of the Symbiotic Patterns of Various Annual Legumes*

The detailed descriptions of symbiosis in field pea and vetch and the observations on other annual species in Northern Ireland and Australia (unpublished work; see also Section III(*c*)) permit the following generalizations to be made for annual legume nodulation studied by the author:

(i) Root growth and attendant nodule initiation are precocious. Maximum numbers of nodules are present on roots by mid- or late-vegetative development, and nodule initiation usually ceases well before flowering.

(ii) In effective symbiosis, nodules acquire haemoglobin pigmentation in ordered sequence on the various portions of the host plant root system. The extent and rate of pigmentation is related to the current growth rate of the host plant.

(iii) Average nodule size and efficiency increase as nodule populations age. There is some evidence of a progressive elimination of the smaller members of effective nodule populations throughout plant growth.

(iv) The commencement of nitrogen fixation in the seedling coincides with the pigmentation of the first few nodules on the primary root. The former event usually anticipates the exhaustion of available cotyledon reserves.

(v) Nitrogen accumulation in host tissues and symbiotic organs proceed such that the maximum in total nodule nitrogen is attained some time before the maximum in total plant nitrogen. This observation reflects the general increase in fixation efficiency in aging nodule populations (see (iii) above).

(vi) There is evidence of an immediate transfer of fixation products from nodules to host plant tissues. At no time in annual legume development do roots or nodules act as substantial nitrogen storage organs on the plant.

(vii) Haemoglobin destruction in senescent nodules progresses from base to apex. Approximately one-third of the nitrogen in the nodule is removed in early senility. It is estimated that in effective symbiosis of the annual legumes field pea and vetch less than 3 per cent. of the nitrogen fixed in the symbiotic cycle is returned to the soil in the form of dead nodule tissue (see Pate 1958 for further discussion).

(viii) Dramatic decreases in nodule numbers and total weight of nodules occur in flowering and fruiting stages of the life cycle. Minor differences are noted in this symbiotic feature for the nodulation performances of vetch and field pea. Root development, nodule initiation, nodule disappearance, and several attendant symbiotic features are earlier in field pea than in vetch when compared on a host plant nitrogen accumulation basis. These differences may constitute host species nodulation characters or may merely reflect compatability differences between host and bacterial partners of the two particular associations examined in this series of investigations.

### (c) Analysis of the "Flowering Factor" in Nodule Destruction in V. sativa

In the experimental series, where nodulation of vetch plants maintained in prolonged vegetative condition by flower-bud removal was compared with the nodulation of similarly aged untreated plants, it is seen that plant flowering inhibits both root growth and nodule initiation while concurrently hastening the destruction of existing nodules. This analysis does not necessarily imply action of two separate physiological effects of flowering. The nutritional demands of flowering may promote withdrawal of protein and carbohydrate from vegetative organs and the consequent starvation of underground portions of the plant may then both arrest root growth and promote rapid nodule emptying. Thus, for the present, the flowering factor in the nodulation cycle may be conveniently relegated to the many little-understood physiological effects associated with annual plant flowering. More detailed analyses of the carbohydrate and nitrogen nutrition of nodules on flowering plants might well lead to a greater appreciation of the actual physiological processes involved in nodule destruction.

The practical implications of delaying or preventing flowering in an annual legume are obvious. For instance, greater final yield with longer growing season could be obtained from the symbiosis of a species grown in climatic conditions unsuited to flowering or seed setting, provided that the symbiotic machinery permitted continued nitrogen fixation throughout the extended vegetative phase. Similarly, cutting of plant tops or light grazing might retard flowering and its accompanying nodule degeneration.

### (d) Nodule Longevity in Various Irish Legumes

The most surprising result of the seasonal nodulation studies in Northern Ireland is that the normal life span of the nodule is not moulded by climatic influences to the extent depicted by previous authors. Successful overwintering of nodules in Ireland is recorded as a common event in each of the legume life forms studied, and it would be interesting to compare these effects with the overwintering of legumes in geographical regions experiencing more prolonged and intense cold in the autumn and winter months. The biennial nodules described for the woody genera *Ulex* and *Sarothamnus* provide further evidence of restricted climatic interference with nodule longevity patterns. In the case of these woody perennials in temperate climates it is suggested that nodules may be classed as "potentially

perennial", i.e. their anatomical and physiological constitution will allow for perennation if suitable climatic conditions persist throughout their life span.

Frost destruction of nodules, where occurring, is clearly incidental and super-imposed on the normal nodulation pattern of the host plant. Thus, the autumn set of young nodules in the winter annual may be partially or completely destroyed by winter conditions, while mature nodules may be destroyed in the overwintering biennial or perennial. One might speculate that other influences such as high soil temperatures, drought, and grazing effects would promote a similar interruption of the normal sequences of nodulation. Study of the effects of these factors would be particularly interesting in the Australian environment.

## V. Acknowledgments

The work described in this paper forms part of a Ph.D. thesis and the author wishes to express his thanks to his supervisor, Professor J. Heslop-Harrison, Queens University, Belfast, for helpful suggestions and criticism. The author is also indebted to Professor R. L. Crocker, Botany Department, University of Sydney, for his helpful criticism of the manuscript.

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# STUDIES OF THE GROWTH SUBSTANCES OF LEGUME NODULES USING PAPER CHROMATOGRAPHY

By J. S. Pate*

*Summary*

Five distinct growth substances active in the *Avena* coleoptile straight-growth test were separated by paper chromatography of ethanol extracts of nodules of *Pisum arvense* L. and *Ulex europaeus* L. Three of the active substances were classed as promotors, two as inhibitors, of coleoptile elongation. They exhibited the following migration behaviour in *iso*propanol–ammonia chromatography solvent:

Accelerator 1 (A$_1$): acidic, $R_F$ 0·05–0·15.
Accelerator 2 (A$_2$): acidic, $R_F$ 0·30–0·45.
Accelerator 3 (A$_3$): non-acidic, $R_F$ 0·70–0·85.
Inhibitor 1 (I$_1$): non-acidic, $R_F$ 0·50–0·60.
Inhibitor 2 (I$_2$): non-acidic, $R_F$ 0·75–0·85.

A$_2$ was identified colorimetrically and by ultraviolet fluorescence as indole-acetic acid. The chemical identity of the other constituents was not determined but similarities in migration and fractionation were noted between A$_1$ and the a-accelerator of Bennet-Clark and Kefford (1953), and between A$_3$ and the indoleacetonitrile of Cruciferae extracts.

In terms of *Avena* response, A$_2$ (indoleacetic acid) was designated as the dominant auxin of *Pisum* nodules, being recovered in large amounts at all stages of nodule growth. A$_1$ first appeared in chromatograms of young, recently pigmented nodules, and thereafter retained a fairly uniform activity until nodule senescence. A$_3$ appeared in quantity during the second week of nodule life, but diminished to minute or undetectable amounts as nodules aged. Both inhibitors were consistent features of tissue extracts and apparently increased in concentration with nodule growth.

There was no observable qualitative or quantitative decrease in *Pisum* nodule growth substances as haemoglobin decomposition took place in senescent nodules.

The meristematic and bacterial portions of the *Ulex* nodule contained approximately similar amounts of the nodule growth substances.

All *Avena*-active substances of nodule extracts except A$_3$ were recorded from assays of *Pisum* root extracts. Extractable auxin levels were found to be very much lower in root tissues than in nodules.

## I. Introduction

In the earliest accounts of symbiosis it was suggested that nodule initiation might be a direct consequence of the diffusion of active substances produced by bacterial synthesis in the infected tissues of the legume root (see Fred, Baldwin, and McCoy 1932; Wilson 1940; Thimann 1955). The present trend of thought still

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ascribes a dominant role to growth substances in nodule genesis, although the best evidence still fails to prescribe the origin and identity of chemical agents specifically responsible for mitotic stimulation in nodule development.

Interest in nodule auxins resulted largely from the researches of Thimann (1936, 1939) and Thimann, Skoog, and Byer (1942) who used the *Avena* coleoptile curvature test to demonstrate that nodules contained diffusible auxins in much greater concentration than neighbouring root tissues. Auxin production in nodules paralleled nodule growth; auxin occurred in excessive amounts only in infected tissues; apical halves of nodules yielded similar amounts of diffusible auxin to basal halves; and degenerate nodules contained little, if any, auxin. Assays of nodules conducted by Link and Eggers (1940) and Egle and Munding (1951) have also described nodule tissues as being very rich in extractable auxins.

The bacteria themselves have been considered to be responsible for auxin syntheses in the nodule. Production of indoleacetic acid by *Rhizobium* in various culture media containing tryptophan has been demonstrated by Link (1937), Chen (1938), Thimann (1939), and Georgi and Beguin (1939).

Thimann (1939) proposed that the physiology of nodule formation was pivoted on the activity of the auxins produced by the nodule bacteria. He depicted nodule genesis as a similar process to lateral root development, but where the maintenance of supraoptimal levels of growth substances prevented elongation of infected tissues into a structure resembling a root. This concept of the nodule as an arrested secondary root has been rejected by Wilson (1940), Bond (1948), and Allen and Allen (1953) who stress that the nodule is a unique type of root hypertrophy in being of cortical origin and of markedly different structure and function from a secondary root.

Application of various growth substances to roots have failed to discover stimulants of cortical proliferation, if such substances exist. Kraus (1941), Bond (1948), and Allen, Allen, and Newman (1953) have all reported negative findings in this respect, although the last-named authors produced pseudonodular outgrowths on legume roots by application of 3, 5-dichlorobenzoic acid, and Bond (1948) described localized vascular differentiation in roots following application of tryptophan.

The extensive investigations by Nutman on the physiology of nodule formation in red clover and other legumes have given adequate evidence of hormone-type interactions in nodulation (see Nutman 1956). Seedling root excretions have been described as influencing the extent and precocity of nodulation in agar cultures of legumes, while characteristic internodular reactions have been demonstrated for later symbiotic development. The chemical identities of the respective active substances are apparently unknown.

The research detailed in this paper was originally designed as a quantitative analysis of auxins in the maturing nodule. Preliminary studies showed marked antagonism between auxins, inhibitors, and interfering impurities in nodule extracts which prevented use of conventional colorimetric or biological assays. Consequently the technique of paper chromatography coupled to *Avena* assay was explored as an alternative method of comparing tissue extracts. This proved satisfactory for a semi-quantitative description of nodule growth substances in aging nodule tissues.

## II. Materials and Methods

### (a) Extraction of Growth Substances

A standard, low-temperature ethanol extraction procedure followed by hexane–acetonitrile purification was used in all analyses described in this paper:

(i) The fresh tissue was ground with half its volume of quartz sand and covered with 10 times its weight of absolute ethanol. Extraction was allowed to proceed for 20 hr at −15°C.

(ii) The extract was filtered, the ground tissue washed three times with small volumes of ethanol, and the combined extract then evaporated to dryness under reduced pressure (temperature kept below 50°C).

(iii) The residue from evaporation was redissolved in 25 ml acetonitrile and partitioned with five washings of 20 ml hexane (B.P. 68°C) (see Nitsch 1953). Trial chromatograms showed that the hexane removed lipoidal impurities while apparently all of the extracted growth substances were retained in the acetonitrile fraction.*

(iv) The acetonitrile layer was reduced to small volume by evaporation under reduced pressure and applied to the chromatogram as a total extract.

(v) In some experiments the purified extract from (iii) was fractionated into acidic and non-acidic portions by the method of carbonate to phosphoric acid transfer given by Bonde (1953).

### (b) Chromatography of Extracts

Ascending–descending chromatography on Whatman No. 1 paper was used with isopropanol–ammonia (sp. gr. 0·880)–water (80 : 5 : 15 v/v). According to Stowe and Thimann (1954) this is the most versatile solvent, and it has been widely used by many workers in the auxin chromatography field. The solvent gave satisfactory separation of the active fractions of nodule tissues. The following chromatography procedure was used: equilibration, 3 hr; running time, 11–15 hr at 24°C; solvent-front coverage, 25–35 cm; position of indoleacetic acid (IAA) marker spot run under these conditions, $R_F$ 0·35–0·45; position of indoleacetonitrile (IAN) marker spot, $R_F$ 0·70–0·80.

Chromatograms were dried by heating for 10–15 min at 35°C. This procedure removed all traces of solvent from the paper. Chromatograms not immediately required for bioassay were stored in an atmosphere of nitrogen in the dark at −15°C.

### (c) Bioassay of Chromatograms

The biological assay method selected was the *Avena* coleoptile straight-growth test as developed and refined for auxin chromatography work by Bentley (1950) and Bentley and Housley (1954).

*Nitsch (1953) recommends double chromatography as an ideal method of eliminating waxes from crude ether or alcoholic extracts. A preliminary run, with water as solvent, is followed by elution of wax-free portions of the paper and a second chromatographic development in normal solvent. This method proved satisfactory in one series of nodule extracts but was eventually discarded as it involved 3 days of chromatography and final activities of extracts were lower than those obtained from the acetonitrile purification method.

(i) *Elution of Chromatography Segments.*—Chromatograms were examined under ultraviolet light to guide selection and sectioning. Each chromatogram was sectioned into 20 equal segments (i.e. each segment representing 0·05 of an $R_F$ unit). This 20-strip analysis was found necessary for the successful delimitation of active areas, particularly in total extracts containing a variety of adjacent growth-inhibiting and growth-promoting substances.

Segments were eluted for 3 hr in 5-ml petri capsules containing 5 ml 3 per cent. sucrose containing citrate–phosphate buffer as recommended by Nitsch (1953). A carbohydrate source was found to be essential in the *Avena* assay to promote suffic- ient control growth for the detection of inhibitor activity on chromatograms.

(ii) *Growth of* Avena *Coleoptiles.*—Svalof Victory oats were soaked for 14–16 hr in tap-water at 10°C and sown in batches of 2000 in large enamel trays (14 by 16 by 2 in. deep) containing 4 l. of wet, sterile river sand. The trays were placed in the dark in a humidity chamber maintained at 25°C. Mesocotyl growth was suppressed by giving emerging coleoptiles a 5-min exposure to daylight 24 hr before harvest. Coleoptiles were harvested 72 hr after sowing.

(iii) *Bioassay Technique.*—The complete bioassay was set up in a dark room under red light. Coleoptiles were selected from the size range showing 15–18 mm growth above the mesocotyl, and were guillotined on a special cutter which removed a 10-mm subapical portion and rejected a 3-mm tip and the basal portion of the coleoptile. Cut coleoptiles were randomly distributed among the dishes of chromato- gram segments until each dish contained ten 10-mm sections. Ten capsules, with a strip of filter paper and 5 ml of 3 per cent. buffered sucrose, were included as medium control series for each bioassay.

The coleoptile sections were grown in darkness for 24 hr at 25°C. At the conclusion of the growth period, the lengths of the 10 sections in each capsule were recorded by measuring enlarged microprojector images of the coleoptile with an opisometer.

### (d) *Presentation of Results*

The *Avena* activity of each chromatogram was expressed in conventional histogram form where mean coleoptile extension of consecutive chromatogram segments was represented as a percentage of the medium control growth. Significance levels were drawn on each histogram at $+2$ times the standard deviation of coleoptile elongation in the 10 dishes of the medium control series. Values on the histograms outside these limits were taken as significant ($P < 0·01$) evidence of the presence of *Avena*-active growth substances.

### III. RESULTS

### (a) *Qualitative Examination of the Growth Substances in Aging Nodules of the Field Pea,* Pisum arvense *L.*

Eight samples of primary root nodules for chromatogram series 1 were taken from soil-grown field pea plants in September 1955 in the sequence shown in Table 1. Effective nodules were selected from the top 4 in. of primary root to confine develop- mental effects to a similarly aged and sized nodule population.

A second series, chromatogram series 2, was designed to check some of the qualitative changes in nodule auxins noted in the 1955 series. A similar sowing of field pea was made and three age groups of nodules selected for detailed study in September 1956:

Set A: young white nodules, 6-, 3-, and 1-g samples from 2-leaf plants (equivalent to the 13-day stage, 1955 series).

Set B: young red nodules, 7-, 4-, 2-, and 1-g samples from 4-leaf plants (equivalent to the 20-day stage, 1955 series).

Set C: older red nodules, 8-, 4-, 2-, and 1-g samples from 8-leaf plants (equivalent to the 32-day stage, 1955 series).

TABLE 1

NODULE SAMPLING FROM FIELD PEA PLANTS, CHROMATOGRAM SERIES 1, SEPTEMBER 1955

| Sample No. | Days after Sowing | Leaves per Plant (age) | Fresh Wt. of Nodule Sample (g) | % of Nodules with Haemoglobin Pigment | Approx. No. of Nodules in Sample |
|---|---|---|---|---|---|
| 1 | 13 | 2 | 8 | 48 | 8010 |
| 2 | 16 | 3 | 8 | 79 | 6650 |
| 3 | 20 | 4·3 | 8 | 98 | 4050 |
| 4 | 24 | 5 | 4 | 100 | 1400 |
| 5 | 28 | 6·9 | 4 | 100 | 890 |
| 6 | 32 | 8·1 | 4 | 100 | 670 |
| 7 | 37 | 9·4 | 4 | 98 | 600 |
| 8 | 42 | 10·4 | 4 | 93 | 410 |

Activity histograms of series 1 and 2 are presented in Figures 1 and 2 respectively. The following tentative conclusions may be drawn from the results of both series:

(i) Five characteristic growth substances can be detected in the *Avena* assay of nodule extracts. The following names and $R_F$ values are assigned to the active areas described on the histograms and subsequently mentioned in the text:

| Growth Substance | $R_F$ at which Maximum Activity Occurs (*iso*propanol–ammonia solvent) |
|---|---|
| Accelerator 1 ($A_1$) | 0·05–0·15 |
| Accelerator 2 ($A_2$) | 0·30–0·45 |
| Accelerator 3 ($A_3$) | 0·70–0·85 |
| Inhibitor 1 ($I_1$) | 0·50–0·60 |
| Inhibitor 2 ($I_2$) | 0·75–0·85 |

(ii) $A_1$ becomes detectable on chromatograms of extracts from nodules of 3-leaf plants (i.e. just after the majority of nodules have turned red). It is recovered from all older nodule samples.
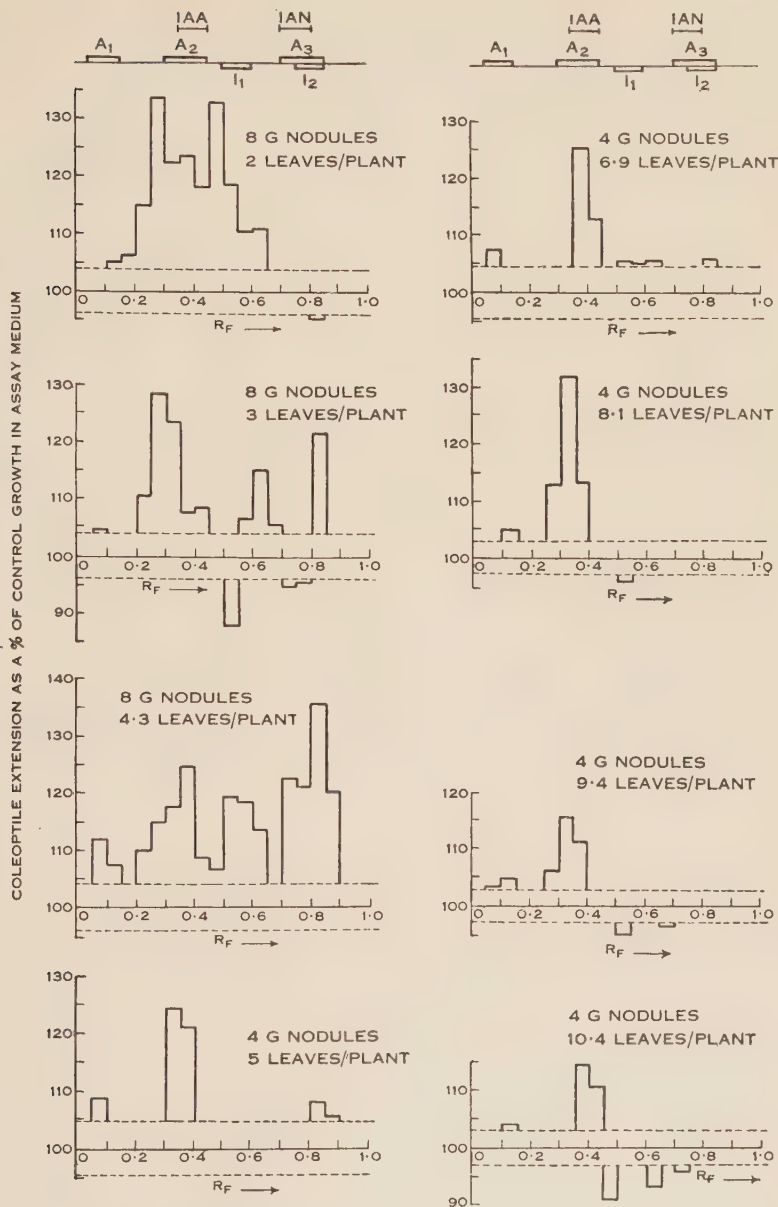
Fig. 1.—Growth substances in aging nodules of *P. arvense*. *Avena*-activity histograms of chromatograms of total alcohol extracts of samples of eight age groups of primary root nodules from soil-grown field pea plants. Details of the nodule samples are given in Table 1. (In the histograms of Figures 1–4 mean coleoptile extensions of consecutive chromatogram segments ($R_F$ scale) are represented as a percentage of coleoptile growth in pure assay medium (vertical scales). Significance levels are drawn at $\pm 2$ times the standard deviation of coleoptile elongation in the medium control series. The positions of marker spots of IAA and IAN and of the typical $R_F$ values assigned to the five nodule growth substances are included at the top of each set of histograms.)

(iii) $A_2$ is present in large amounts at all stages of nodule development. There is evidence of supra-optimal activity in the $A_2$ zone in several histograms (e.g. the 8-g samples of series 1 (Fig. 1) and 7-g sample of series 2 (Fig. 2)). In series 2 there
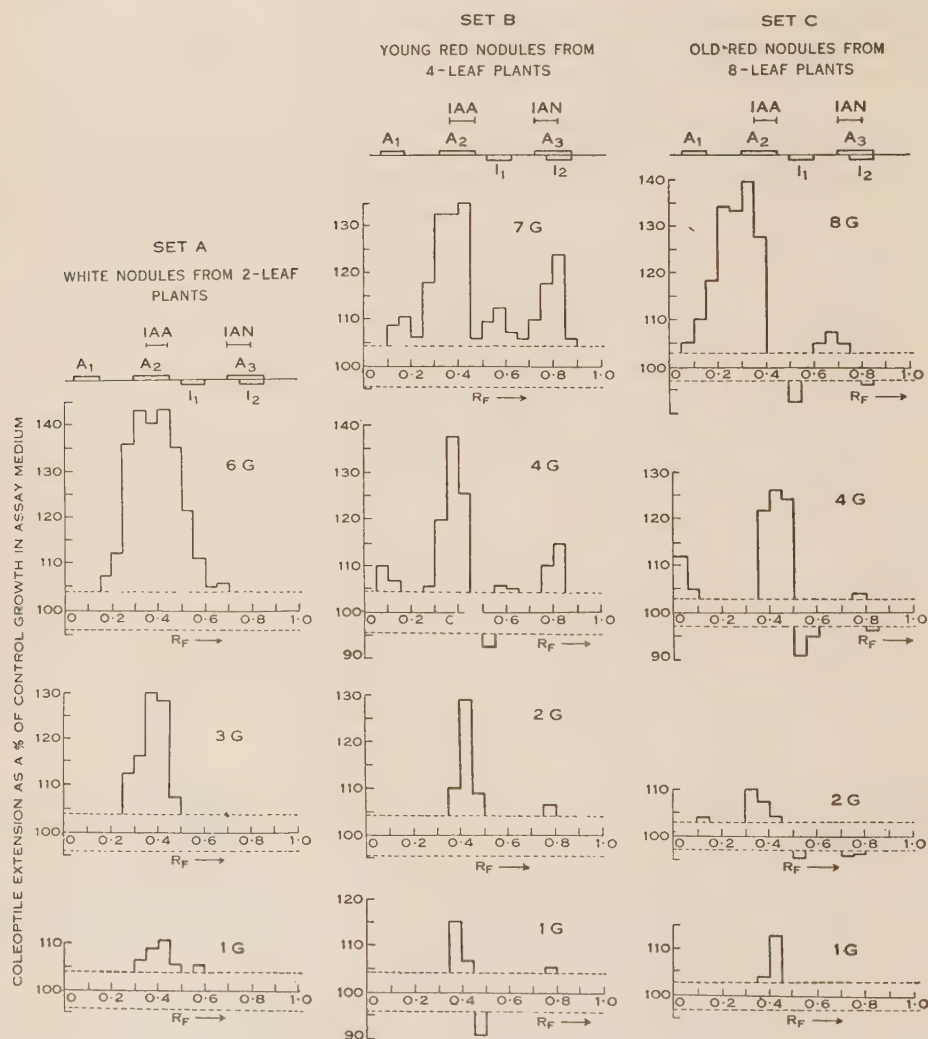


Fig. 2.—Growth substances in aging nodules of *P. arvense*. *Avena*-activity histograms of chromatograms of total alcohol extracts of varying quantities of tissue from three age groups on nodules (chromatogram series 2, see text).

is a fairly close relationship between amount of material assayed and *Avena* activity in the $A_2$ region, with $A_2$ detectable even from 1-g quantities of nodules. There is some evidence of a slight decrease in $A_2$ concentration in the aging nodule, particularly after 5 weeks of nodule growth.

(iv) $A_3$ is absent from very young nodules but appears in quantity in the samples from 4-leaf plants. Thereafter $A_3$ activity diminishes until, at the 8-leaf stage of plant development, it is barely detectable from nodule tissues.

(v) It is difficult to provide reliable information on fluctuations in the *Avena*-inhibitory substances of the nodule. $I_1$ may be masked by overlap with high $A_2$ concentrations, and $I_2$ may be similarly antagonistic to $A_3$ activity. These antagonisms are demonstrated clearly in the series 2 chromatograms (Fig. 2). The sporadic occurrence of $I_1$ and $I_2$ throughout the samples would indicate that both are a consistent feature of tissue extracts. There is some evidence from both series that inhibitors are more concentrated in older nodules.

(vi) The three stages examined in series 2 confirm the auxin changes described in series 1 and also provide evidence of a change in relative concentration of the three accelerators. $A_2$ is the only accelerator visible in bioassay of extracts of young white nodules; young red nodules have the three accelerators present in relative proportions $A_2 > A_3 > A_1$; in older nodules the order of concentration changes to $A_2 > A_1 > A_3$.

### (b) Further Characterization of Nodule Growth Substances

(i) *Chemical Identification of Nodule Growth Substances.*—A 250-g nodule sample from *Pisum* plants was used for this analysis. The purified extract was run as a long strip on the chromatogram. Strips of the developed chromatogram were treated with ferric–perchloric reagent* and with Ehrlich's reagent† and gave characteristic IAA colour reactions in the $0\cdot30$–$0\cdot50$ $R_F$ region. Ultraviolet illumination of untreated strips showed typical ash-coloured fluorescence in the same $R_F$ region giving additional confirmation of the presence of IAA in nodule tissues. Identifiable colorimetric and fluorescence reactions could not be obtained from other regions of the chromatograms.

(ii) *Fractionation of Nodule Growth Substances into Acidic and Non-acidic Constituents.*—Figures 3(*a*) and 3(*b*) detail sample activity bioassay histograms of chromatograms of acidic and non-acidic fractions of nodule extracts. Equal weights (3 g) of apical and basal portions of nodules of *Ulex* were assayed (Fig. 3(*a*)). There was no evidence of differences between the constituents of the two halves of the nodules. $A_1$ and $A_2$ accumulated in the acid fractions, $I_1$ and $I_2$ in the non-acidic fractions. Figure 3(*b*) shows a typical activity histogram following fractionation of extracts of young *Pisum* nodules. $A_1$ and $A_2$ are again designated as acidic substances and $I_1$, $I_2$, and $A_3$ are non-acidic. In both series there is evidence of a slight IAA "leak" into the non-acidic fraction.

(iii) *Growth Substances in the Senescent Nodule.*—Several extracts of red and green nodules of similar age were compared in both *Pisum arvense* and *Ulex europaeus*. A typical set of histograms is depicted in Figure 3(*c*). All the activity zones except $A_3$ are reproduced in green and red samples and there is no evidence of any extensive destruction of any of the growth substances coincident with haemoglobin destruction in early nodule senility.

*50 parts of 5 per cent. perchloric acid to 1 part $0\cdot05$M ferric chloride.

†1 per cent. *p*-aminobenzaldehyde in 1N hydrochloric acid.
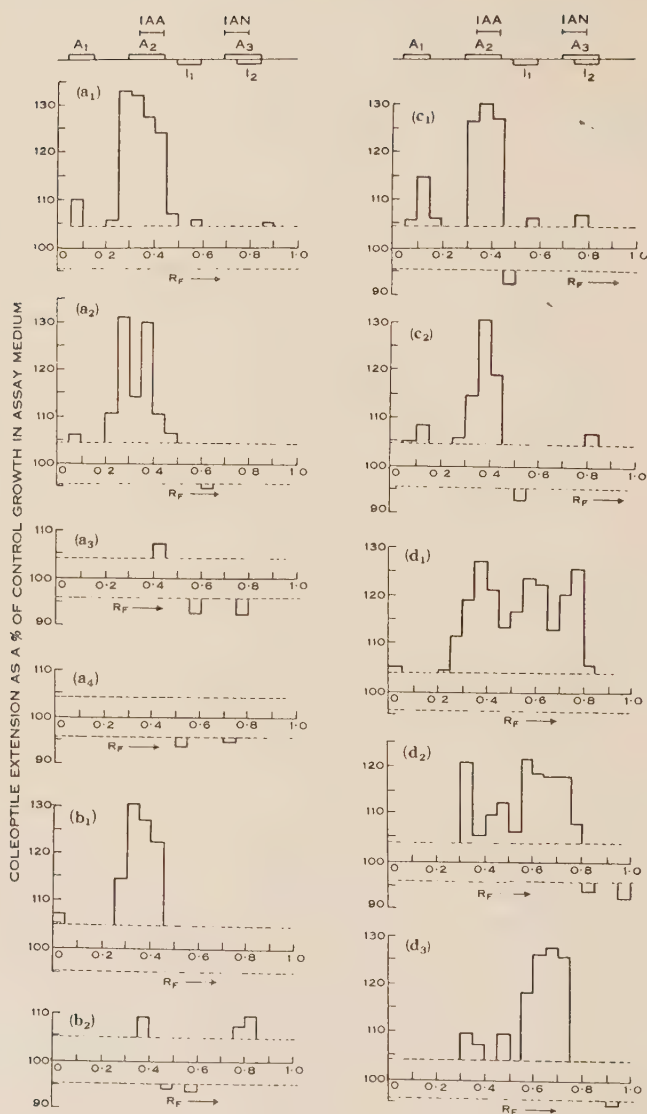
Fig. 3.—$(a_1)$–$(a_4)$ *Avena*-activity histograms showing typical comparisons of chromatograms of the acid and non-acid fractions of alcohol extracts of basal and apical portions of 1-year-old nodules of *Ulex europaeus*. $(a_1)$ 3 g apical portions, acid fraction; $(a_2)$ 3 g basal portions, acid fraction; $(a_3)$ 3 g apical portions, non-acid fraction; $(a_4)$ 3 g basal portions, non-acid fraction. $(b_1)$, $(b_2)$ Comparison of the *Avena*-activity histograms of chromatograms of the acid $(b_1)$ and non-acid $(b_2)$ fraction of an alcohol extract of 5 g of red nodules of *P. arvense*. $(c_1)$, $(c_2)$ Comparison of the *Avena*-activity histograms of chromatograms of total alcohol extracts of 4-g quantities of red $(c_1)$ and green $(c_2)$ nodules from 84-day-old plants of *P. arvense*. $(d_1)$-$(d_3)$ *Avena*-activity histograms of chromatograms of total alcohol extracts of 40 g $(d_1)$, 20 g $(d_2)$, and 10 g $(d_3)$ of Brussels sprouts tissue.

(iv) *Comparison of Nodule and Brussels Sprouts Extracts.*—The activity histograms of 40-, 20-, and 10-g portions of Brussels sprouts tissue (*Brassica oleracea*) are included in Figure 3(*d*) solely for comparative purposes. Marked activity in the IAN region is noted in the same position as the $A_3$ activity of nodules. Activity in the $A_3$ region is represented in this Cruciferae extract in apparently more active proportions than is the $A_2$ (? IAA) activity zone.
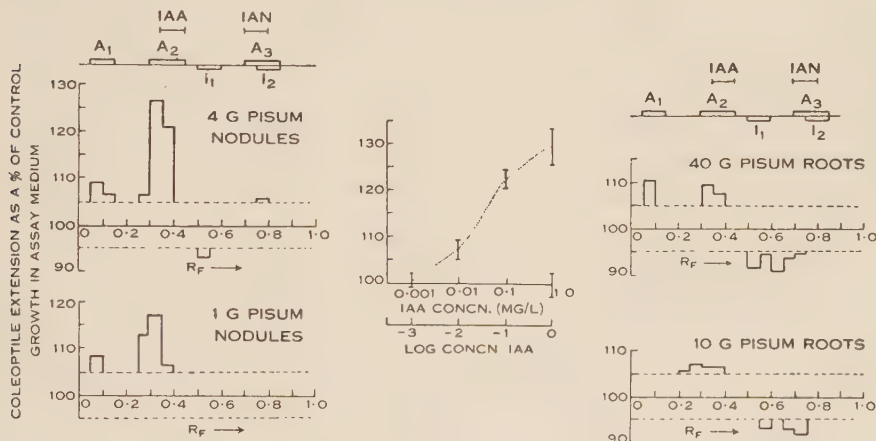


Fig. 4.—Typical activity histograms of chromatograms of total alcohol extracts of nodule and root tissues from 54-day-old plants of *P. arvense*. A calibration curve of coleoptile elongation against the logarithm of IAA concentration is included for comparison of the extractable auxin levels in the two tissues (see text).

(v) *Comparison of Nodule and Root Extracts in* Pisum.—Growth substance activities of 10 and 40 g of *Pisum* roots are recorded in Figure 4. Root auxin levels are seen to be very much lower than in nodules, although all of the five zones showing *Avena*-activity zones, except $A_3$, are recorded for roots. Activity of 1-g and 4-g *Pisum* nodule extracts can be compared with the root extract activities by reference to a calibration curve of coleoptile extension against the logarithm of IAA concentration. It is estimated that IAA-extractable activity in roots is some 40–60 times lower than in nodule tissues of similar age (c.f. similar report by Thimann, Skoog, and Byer 1942).

High concentrations of $I_1$ and $I_2$ are recorded for root tissues (cf. reports of inhibitor action in roots by Howell 1954; Torrey 1956).

## IV. Discussion

Chromatograms of ethanol extracts of *Pisum* and *Ulex* nodules and *Pisum* roots show five characteristic zones of activity in the *Avena* straight-growth test. Of these, three are classed as promoters ($A_1$, $A_2$, $A_3$) and two as inhibitors ($I_1$, $I_2$) of coleoptile elongation.

$A_2$ is identified chemically as indoleacetic acid (IAA) confirming previous reports of the presence of this growth substance in legume nodules by other workers. The chemical identity of the other four constituents cannot be specified. The $R_F$ and

fractionation behaviour of $A_1$ are similar to those of the α-accelerator described by Bennet-Clark and Kefford (1953). This substance has been described for a variety of tissues including legume roots (see Kefford 1955). On similar evidence, $A_3$ may be associated with indoleacetonitrile (IAN), the neutral component which has been isolated from Cruciferae extracts (Jones *et al.* 1952). Unfortunately, owing to the small amounts in nodule tissues, it was not possible to hydrolyse $A_3$ zones on chromatograms to see if IAA was produced, as would be expected from an IAN source. Of the two inhibitors, $I_1$ has similar migration behaviour to the β-inhibitor of Bennet-Clark and Kefford (1953), yet $I_1$ is recorded as a non-acidic substance. $I_2$ cannot be identified with any "named" activity area mentioned by other authors. However, large amounts of *Avena*-inhibitory substance have been recorded from this high $R_F$ region from extracts of bean plumules (*Phaseolus*) and stolons of blackberry (*Rubus*) (Gunning and Pate, unpublished data.)

The qualitative analysis of growth substances in developing *Pisum* and *Ulex* nodules depicts an ordered sequence of auxin changes, but fails to provide any basis for connecting these events with the fundamental growth pattern of the nodule. The usual criticisms of this type of auxin assay apply here. It is not known whether the ethanol extraction method used here is a wholly satisfactory method of estimating diffusible auxin activity: interconversions between indole compounds are known to occur in some extraction procedures. Are the extracted substances identified as being active in monocotyledon cell elongation of definite function in the tissues of the legume nodule, or even present in the nodule in suitable condition for physiological activity?

Despite the above limitations on interpretation the results demonstrate that nodule tissues are centres of very intensive growth substance activity, particularly IAA activity. In all probability the nodule is self-contained in its auxin syntheses. As yet it is not known whether nodule auxins are metabolic products of the microsymbiont, substances released by host cells in response to bacterial irritation, or, as is more likely, represent a synergy of host and bacterial processes. In the latter case it might be held that the rhizobia were supplied with suitable precursors from host cell proteolytic activities.

The presence of similar growth substances in root and nodule tissues would suggest the presence of similar synthetic mechanisms within these organs, though it is difficult to explain why there are such large quantitative differences in the diffusible auxin contents of nodule and root. Oxidative enzymes may be absent or blocked, as suggested by the work of Wagenknect and Burris (1950), or high levels of auxin may accumulate in nodules simply because normal oxidase systems cannot cope with the intensive and continued synthetic activities of the intracellular bacteria.

A further question is whether there is a substantial backward diffusion of nodule growth substances into parent root tissues. If there is, there may be a mechanism of root inactivation of these substances, possibly by oxidase systems or by acceptance into bound complexes. Nutman's (1956) studies give evidence of internodular inhibitions elicited by excretions from nodule meristems. It is tempting to suggest that these inhibitory effects might be connected with a backward diffusion of nodule

auxins and inhibitors. Further observations of internodular reactions will be provided in a further paper on the effect of delayed inoculation on legume nodulation.

In the developmental sequences of growth substance activity in nodule growth, IAA ($A_2$) emerges as the major *Avena*-active auxin in the nodule. It is present in large quantities at all stages of nodule growth, even until nodules have turned green in early senility. The general effect of this auxin may be one of supra-optimal inhibition of meristem elongation as originally suggested by Thimann (1939). If so, one might expect nodules to elongate if suitably relieved from hyperauxomy early in their growth. This has not been achieved. IAA is the only auxin detectable in very young nodules and it may well be of function in the mitotic stimulation of early nodule development (see Thimann 1955). In this connection high levels of IAA have been recorded from various fungal and bacterial galls and tumours which also exhibit pronounced tissue proliferation unaccompanied by normal cell elongation.

Coincident with nodule pigmentation, two further accelerators become detectable in nodule extracts, one ($A_1$) to remain as a constant feature of tissue extracts, the other ($A_3$) to diminish markedly in subsequent nodule growth. Later in nodule development the two inhibitors, $I_1$ and $I_2$, apparently increase in nodule tissues. What do these fluctuations in minor constituents represent? The well-defined $A_3$ maximum may be singled out as possibly important as a temporary reserve of indole material for subsequent auxin releases in the nodule.

A biochemical approach is obviously required to add further to the present picture. In the first place this might be aimed at discovering by what systems and with what materials the high levels of the various growth substances are elaborated and maintained in the nodule. Secondly, the physiological implications of high levels of indole compounds in the nodule might be examined, particularly with reference to possible interaction with other classes of growth substances, e.g. the kinins and gibberellins. The results from such an approach might well pave the way to further study of the basic problem of how nodule growth is synchronized with general host plant development.

## V. Acknowledgments

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# FREE AND BOUND AMINO ACIDS IN LEGUME ROOT NODULES: BOUND γ-AMINOBUTYRIC ACID IN THE GENUS *TRIFOLIUM*

By G. W. Butler* and N. O. Bathurst*

## Summary

"Free" and "bound" amino acids in root nodules from 10 legume species have been studied. In general, the free amino acids comprise a small proportion (25 per cent. or less) of the total nitrogenous compounds soluble in 80 per cent. (v/v) ethanol.

Bound amino acids were present in substantial amounts in the nodules from all 10 legumes.

In particular, "bound γ-aminobutyric acid" was present in large amounts in nodules of *Trifolium repens* and in smaller amounts in *T. pratense* and *T. medium*.

In *T. repens*, bound γ-aminobutyric acid was present only in effective nodules, being absent from leaf, stem, and root tissues, from ineffective nodules, and from *Rhizobia* in liquid culture.

Some properties of bound γ-aminobutyric acid are described.

## I. Introduction

Steward and Thompson (1950) have suggested that the composition of the non-protein-nitrogen fraction of nodules should be further elucidated. This paper deals with the amino acids which can be found in "free" and "bound" form in protein-free extracts from legume nodules. In the particular case of nodules from white clover (*Trifolium repens*), the presence of considerable amounts of bound γ-aminobutyric acid is reported. A preliminary report of part of this work has been given elsewhere (Butler and Bathurst 1957).

## II. Methods

### (a) Sampling and Extraction

Roots, together with nodules, were removed from plants growing freely in the field in association with other species and immediately freeze-dried, after which the nodules, consisting of specimens of all ages and sizes, were dissected out and cleaned by sieving followed by dusting with a camel-hair brush. Solubility in 80 per cent. (v/v) ethanol was used as the criterion for the presence of nitrogenous substances of low molecular weight. Extracts were made by homogenizing nodule tissue (100 mg dry weight) with 25 ml 80 per cent. ethanol at room temperature, followed by centrifugation for 5 min at 2000 *g*. The supernatant was concentrated to dryness by means of a rotary vacuum drier, taken up in 1 ml water, and clarified by centrifugation at 5000 *g*.

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## TABLE 1

### FREE AND BOUND AMINO ACIDS IN ETHANOL EXTRACTS OF NODULES OF LEGUMINOUS PLANTS

Amino acids (expressed as mg amino nitrogen per 100 g dry wt.) in ethanol-soluble fraction before and after hydrolysis with 6N HCl for 16 hr

| Amino Acid | T. repens | | T. pratense | | T. medium | | Lotus uliginosus | | Lotus corniculatus | | Pisum sativum | | Cytisus scoparius | | Lupinus angustifolium | | Galega officinalis | | Medicago lupulina | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After |
| Aspartic acid | 6 | 101 | 4 | 136 | 16 | 108 | Trace | 42 | Trace | 25 | 7 | 56 | 32 | 236 | 39 | 149 | 5 | 175 | 7 | 165 |
| Asparagine | 72 | — | 104 | — | 82 | — | 12 | — | Trace | — | 55 | — | 116 | — | 14 | — | 158 | — | 173 | — |
| Glutamic acid | 4 | 37 | 12 | 22 | 28 | 28 | 17 | 29 | 6 | 13 | 6 | 18 | 8 | 68 | 21 | 143 | 32 | 46 | 4 | 25 |
| Glutamine | — | — | Trace | — | — | — | — | — | — | — | Trace | — | — | — | 3 | — | — | — | — | — |
| Glycine | Trace | Trace | Trace | 3 | Trace | Trace | Trace | Trace | — | Trace | Trace | 20 | Trace | 28 | Trace | Trace | — | Trace | Trace | Trace |
| Alanine | 13 | 31 | 24 | 25 | Trace | Trace | Trace | 18 | Trace | Trace | 2 | 30 | 4 | 68 | 28 | 38 | 6 | 11 | 5 | Trace |
| Valine | Trace | Trace | Trace | 9 | — | — | Trace | 14 | Trace | Trace | 1 | 6 | — | Trace | Trace | 12 | Trace | 11 | Trace | Trace |
| Isoleucine | Trace | Trace | Trace | 18 | — | — | Trace | Trace | Trace | Trace | 2 | 5 | — | Trace | Trace | 15 | Trace | 19 | Trace | Trace |
| Serine | 6 | 6 | 4 | 11 | Trace | Trace | Trace | 21 | Trace | 13 | 2 | 6 | 3 | 8 | * | 11 | 3 | 7 | Trace | 76 |
| Threonine | Trace | Trace | 4 | 8 | — | — | — | — | — | — | — | — | — | 18 | — | 12 | 4 | 13 | — | Trace |
| γ-Aminobutyric acid | 2 | 1006 | 12 | 120 | — | 104 | — | 29 | — | Trace | 12 | 18 | 8 | 12 | 11 | 12 | 13 | 6 | Trace | Trace |
| Others | Trace | — | 16 | 28 | — | — | Trace | 15 | Trace | 35 | 32 | 53 | 70 | 100 | 46 | 69 | 108 | 127 | 20 | 45 |
| Total amino nitrogen | 103 | 1181 | 180 | 380 | 126 | 240 | 29 | 167 | 6 | 86 | 119 | 212 | 241 | 538 | 162 | 461 | 329 | 415 | 209 | 311 |
| Total ethanol-soluble nitrogen (mg % dry wt.) | 1390 | | 736 | | 740 | | 358 | | 358 | | 619 | | 1840 | | 1075 | | 1350 | | 989 | |

* Included with glutamic acid.

## (b) Chemical Determinations

(i) *Total Nitrogen.*—This was determined by the micro-Kjeldahl procedure. The extracts were first spot-tested with a 0·1 per cent. solution of diphenylamine in conc. $H_2SO_4$ for the presence of nitrate. Where nitrate was present, a preliminary reduction step, using 20 mg reduced iron in 3 ml 30 per cent. $H_2SO_4$, was carried out prior to the micro-Kjeldahl digestion.

(ii) *Free Amino Acids.*—These were determined by two-dimensional paper chromatography. For each extract, suitable aliquots were applied to Whatman No. 1 papers and chromatograms run at 20°C, using butanol–acetic acid–water (25 : 6 : 25 by volume) in one direction followed by water-saturated phenol in the other. The papers were dried in a current of slightly warmed air and sprayed with a solution of 1 per cent. ninhydrin in 95 per cent. (v/v) ethanol containing 1 per cent. collidine. They were placed in the dark for 24–48 hr at 40 per cent. relative humidity and 20°C for full development of the colours. The intensities of the coloured spots were measured photometrically according to Wellington's modification (1952, 1953) of the method of Thompson, Zacharias, and Steward (1951) and Thompson and Steward (1951). Standard curves were prepared for each amino acid so that the amount of each could be read off. For unknown amino acids a curve giving average amino nitrogen values was used.

.(iii) *Bound Amino Acids.*—To determine the amino acids released by hydrolysis of ethanol extracts, suitable aliquots were refluxed in 6N HCl for 16 hr. Excess HCl was removed by evaporating the extract down to dryness two to three times from distilled water, the pH being finally brought to 6·7. The residue was taken up in 80 per cent. ethanol, the ethanol removed *in vacuo*, and the aqueous solution finally made to the same volume as the original aliquot. Amino acids were then estimated by paper chromatography, as above.

## III. Results

### (a) Free and Bound Amino Acid Content of Nodules

In Table 1 are presented the results of analyses of the ethanol-soluble fraction of nodules from 10 legumes for free amino acids and for amino acids present after hydrolysis with 6N HCl for 18 hr. The data for each plant refer to a single harvesting of nodules. Since the harvests were made from plants growing under a variety of field conditions at differing times of the year, detailed quantitative comparisons of the data for different plants would be invalid. The following conclusions can, however, be drawn:

(1) The free amino acids, in general, comprised a small proportion (up to 25 per cent.) of the total ethanol-soluble nitrogen. Asparagine was present in comparatively large amounts in the *Trifolium* species examined, in *Medicago lupulina*, and in *Galega officinalis*. In the latter species a particularly high proportion of the ninhydrin-positive nitrogen was unidentified; it was mainly present in two spots whose $R_F$ values in phenol–water and butanol–acetic acid–water respectively were 0·18, 0 and 0·33, 0.

(2) In all species, the occurrence of bound amino acids in the ethanol-soluble fraction could be demonstrated by hydrolysis with 6N HCl. In most cases there was still a considerable gap between the sum of free and bound amino acids and the total ethanol-soluble nitrogen. This is partly accounted for by ammonia nitrogen liberated from asparagine by hydrolysis. In white clover nodules, however, γ-aminobutyric acid was formed in large quantities on acid hydrolysis and accounted for the major portion (about 70 per cent.), of the ethanol-soluble nitrogen. Bound γ-aminobutyric acid was also present in comparatively large amounts in *T. pratense* and *T. medium*; it was not present in significant amounts in the nodules of other species examined.

Of the unidentified ninhydrin spots on chromatograms of acid hydrolysates, most were probably basic amino acids.

### (b) Bound γ-Aminobutyric Acid in Clover Nodules

(i) *Location of Bound γ-Aminobutyric Acid on Paper Chromatograms.*—When two-dimensional chromatograms were prepared, two to three small spots, which gave a very weak reaction with ninhydrin, were observed at $R_F = 0$–$0 \cdot 05$ in butanol–acetic acid–water and $R_F = 0 \cdot 80$–$1 \cdot 00$ in phenol–water. One-dimensional chromatograms of a white clover nodule extract applied to 1-in. bands were therefore run and sectors were eluted and analysed for total nitrogen. Blank spaces on each chromatogram were also eluted to give paper blanks for each sector. The results of such an experiment are shown in Table 2

The recovery of the applied nitrogen from sectors of the phenol chromatograms totalled 93 per cent., of which 73 per cent. was present at $R_F = 0 \cdot 70$–$0 \cdot 90$. Recoveries of only 60 per cent. could be obtained from the butanol–acetic acid chromatograms, indicating incomplete elution of the bound γ-aminobutyric acid after chromatography, possibly due to chemical modification during running in the acidic solvent. Of the 60 per cent. of nitrogen recovered, two-thirds was present at $R_F = 0$–$0 \cdot 10$.

In order to obtain bound γ-aminobutyric acid relatively free from amino acids, 1 g dry weight white clover nodules was extracted by the method described above and made to a final volume of 5 ml. The extract was applied in 16-in. bands to four sheets of Whatman No. 1 paper and run in phenol–water. There was good separation with no overloading. Three fluorescent bands were observed under ultraviolet light and strips were cut from each paper comprising the zone $R_F = 0 \cdot 80$–$0 \cdot 90$, this being just below the lowest fluorescent band. The strips were extracted with three changes of water at room temperature, each extraction taking 10 min. The combined extract was reduced in volume by freeze-drying, and the preparation used in the tests detailed below.

(ii) *Reaction of Bound γ-Aminobutyric Acid to Spray Reagents.*—The following spray reagents and tests were used on two-dimensional chromatograms in phenol–water and butanol–acetic acid–water and on one-dimensional chromatograms in phenol–water:

*Ninhydrin.*—Different preparations of bound $\gamma$-aminobutyric acid gave a negative reaction to ninhydrin after running in phenol–water. When run in butanol–acetic acid followed by phenol–water, some preparations gave a faint reaction to ninhydrin, the spot tailing from $R_F = 0 \cdot 90$–$0 \cdot 70$ in phenol–water and $R_F = 0 \cdot 05$ in butanol–water.

*Tests for Guanido Derivatives.*—The extended ninhydrin (Steward, Zacharias, and Pollard 1955) and Sakaguchi (Roche, Thoai, and Hatt 1954) tests were both negative.

*Ureide Test.*—On spraying with dimethylaminobenzaldehyde reagent (Fink *et al.* 1956), a faint yellow spot was observed at $R_F = 0 \cdot 75$ in phenol–water and $R_F = 0 \cdot 57$ in butanol–acetic acid–water. This was ascribed to urea, which was present to the extent of $13 \cdot 2$ mg per cent. nitrogen in the nodules, as assayed by the method of Conway (1957).

TABLE 2

DISTRIBUTION OF THE 80 PER CENT. ETHANOL-SOLUBLE NITROGEN FRACTION OF WHITE CLOVER NODULES ON ONE-DIMENSIONAL PAPER CHROMATOGRAMS

Results expressed as a percentage of the nitrogen applied

| $R_F$ | Solvent Systems | |
| --- | --- | --- |
| | Phenol–Water (saturated) | Butanol–Acetic Acid–Water (25 : 6 : 25 v/v) |
| 0    –0·10 | 0 | 46·2 |
| 0·10–0·20 | 1·5 | 8·4 |
| 0·20–0·30 | 1·5 | 1·5 |
| 0·30–0·40 | 4·3 | 1·2 |
| 0·40–0·50 | 7·0 | 0 |
| 0·50–0·60 | 0 | 0 |
| 0·60–0·70 | 3·0 | 0 |
| 0·70–0·80 | 33·4 | 1·0 |
| 0·80–0·90 | 39·2 | 0 |
| 0·90–1·00 | 2·9 | 2·0 |
| Nitrogen recovered (%) | 92·8 | 60·3 |

(iii) *Chromatographic Identification of $\gamma$-Aminobutyric Acid.*—Suitable aliquots of bound $\gamma$-aminobutyric acid were hydrolysed with 6N HCl for 18 hr as described earlier. Superposition tests of equivalent amounts of authentic $\gamma$-aminobutyric acid and the test material on one- and two-dimensional chromatograms in butanol–acetic acid–water and phenol–water indicated their chromatographic identity. The test material did not complex with copper carbonate impregnated on the paper chromatograms in the path of the compounds during the first solvent movement, whereas $\alpha$-amino acids characteristically do so (Crumpler and Dent 1949). Also tests with $\alpha$-alanine, $\beta$-alanine, ethanolamine, and $\alpha$-aminobutyric acid showed that these compounds moved to positions clearly distinct from the test material.

β-aminobutyric and β-aminoisobutyric acids showed almost the same $R_F$ values as the test material, but reacted more slowly with ninhydrin. The colours of spots developed from the test material and γ-aminobutyric acid were identical using isatin and alloxan spray reagents (Seifer and Oreskes 1956). On paper chromatographic evidence the test material could therefore be identified as γ-aminobutyric acid.

   (iv) *Efficiency of Extraction of Bound γ-Aminobutyric Acid.*—In Table 3, the amounts of free and bound amino acids extracted from freeze-dried white clover nodules by 80 per cent. ethanol and by distilled water respectively at room temperature are presented. Nodules (0·2 g dry weight) were extracted with 25 ml solvent in each case.

TABLE 3

FREE AND BOUND AMINO ACIDS EXTRACTED BY 80 PER CENT. ETHANOL AND WATER
RESPECTIVELY FROM WHITE CLOVER NODULES

Results expressed as mg per cent. nitrogen

| Amino Acid | Ethanol | | Water | |
|---|---|---|---|---|
| | Unhydrolysed | Hydrolysed | Unhydrolysed | Hydrolysed |
| Aspartic acid | 6 | 70 | 14 | 172 |
| Asparagine | 99 | — | 105 | — |
| Glutamic acid | 8 | 18 | 24 | 122 |
| Glycine | — | 43 | — | 215 |
| Alanine | 9 | 11 | 19 | 74 |
| Valine | Trace | Trace | 7 | 96 |
| Isoleucine | Trace | Trace | 8 | 59 |
| Serine | 4 | 11 | — | 67 |
| Threonine | Trace | 6 | 20 | 58 |
| γ-Aminobutyric acid | 20 | 337 | 23 | 1130 |
| Unidentified | 26 | 42 | 159 | 153 |
| Totals | 172 | 538 | 379 | 2146 |
| Total soluble nitrogen | | 787 | | 3062 |

   Of the free amino acids, most were extracted by 80 per cent. ethanol; in the water extract a large ninhydrin-positive spot at $R_F = 0·01$ in butanol–acetic acid and 0·45 in phenol–water accounted for two-thirds of the unidentified ninhydrin-positive nitrogen. After hydrolysis, γ-aminobutyric acid was predominant in both extracts, more being present in the water extract than in the ethanol. Bound amino acids generally were present at higher levels in the water extract. When extractions of the same sample of freeze-dried nodules were made on separate occasions, it was found that bound γ-aminobutyric acid was extracted in varying amounts by 80 per cent. ethanol at room temperature, showing that it was incompletely soluble in 80 per cent. ethanol. Other bound amino acids were much less soluble in 80 per cent. ethanol.

(v) *Stability of Bound γ-Aminobutyric Acid in Acid.*—The stability to acid hydrolysis was investigated by refluxing aliquots of bound γ-aminobutyric acid extract with:

(1) $0 \cdot 1$M borate buffer at pH $6 \cdot 5$ for 2 hr.

(2) N HCl for 2 hr.

(3) 6N HCl for 16 and 24 hr.

Two-dimensional paper chromatograms were run using the hydrolysates. Negligible amounts of γ-aminobutyric acid were liberated at pH $6 \cdot 5$ and $100°C$ for 2 hr. 10–16 per cent. of the bound γ-aminobutyric acid was hydrolysed to γ-aminobutyric acid by N HCl at $100°C$ for 2 hr. Complete hydrolysis was achieved in 6N HCl at $100°C$ for 16 and 24 hr, as assessed by the amount of γ-aminobutyric acid formed.

TABLE 4

DISTRIBUTION OF BOUND γ-AMINOBUTYRIC ACID IN TISSUES OF WHITE CLOVER PLANTS AND IN RHIZOBIA

Unless otherwise stated, data are expressed as mg nitrogen per 100 g dry wt.

| Tissue | γ-Aminobutyric Acid | | Ninhydrin-positive Nitrogen | Total Soluble Nitrogen |
|---|---|---|---|---|
| | Free | Bound | | |
| Effective nodules | $1 \cdot 6$ | 1004 | 1227 | 1390 |
| Ineffective nodules | $1 \cdot 8$ | $6 \cdot 9$ | 159 | — |
| *Rhizobia* in culture | $0 \cdot 1$* | $0 \cdot 0$ | $9 \cdot 0$* | — |
| Roots | $3 \cdot 0$ | $0 \cdot 0$ | 41 | 123 |
| Stems and petioles | $2 \cdot 2$ | $0 \cdot 0$ | 32 | 134 |
| Leaves | $2 \cdot 7$ | $5 \cdot 3$ | 79 | 249 |

* Expressed as mg nitrogen per 100 g fresh wt.

Measurements of the ammonia present after hydrolysis with 6N HCl by the method of Conway (1957) showed that ammonia nitrogen was formed in small quantities (*c.* 5 per cent.) relative to the γ-aminobutyric acid nitrogen.

(vi) *Distribution of Bound γ-Aminobutyric Acid in White Clover Plants.*— In Table 4 are summarized the results of analyses for free and bound γ-aminobutyric acid in ethanol extracts of leaves, stems, petioles, roots, and nodules of white clover plants. The plants sampled were growing vigorously in a ryegrass–white clover pasture in a soil of low nitrogen status. The clover roots were heavily nodulated and root tissue contiguous to nodules was chosen for analysis. It will be seen that the amount of bound γ-aminobutyric acid in effective root nodules of white clover is greatly in excess of the negligible amount present in other tissues of the plant.

In order to ascertain whether bound γ-aminobutyric acid was present in ineffective nodules of white clover, plants were grown in sterile pumice sand and inoculated with an ineffective *Rhizobium* strain. The amounts of free and bound

γ-aminobutyric acid present in the nodules are shown in Table 4; bound γ-amino-butyric acid was present in quantities less than 1 per cent. of that present in effective nodules.

A strain of *Rhizobium* effective for white clover was grown in a medium containing 10 g mannitol, 4 g yeast extract, $0 \cdot 5$ g $KH_2PO_4$, $0 \cdot 2$ g $MgSO_4.7H_2O$, $0 \cdot 1$ g NaCl, and $3 \cdot 0$ g $CaCO_3$ per litre. The bacteria were washed once in water by centrifugation and extracted by homogenizing in 80 per cent. (v/v) ethanol at room temperature. Analyses given in Table 4 indicate that bound γ-aminobutyric acid was not present in this material.

## IV. DISCUSSION

Of the free amino acids present in the various nodule extracts, glutamine was observed only in lupin nodules, whereas asparagine was present, usually in relatively large amounts, in nodules from all species examined. Bathurst (1954) using the non-specific hydrolytic method (Vickery *et al.* 1935) for estimating glutamine, reported higher values for glutamine in lupin nodules. Whereas Hunt (1951) and Virtanen and Miettinen (1953) reported the presence of glutamine in various legume nodules, Sen and Burma (1953) did not report it in a study of the amino acids in nodules of four legume species.

Compared with other plant tissues, free amino acids in nodules of the 10 species examined comprise a small proportion of the total nitrogenous compounds soluble in 80 per cent. ethanol. It is clear that elucidation of the nature of the other nitrogenous compounds present may well be of significance in studies of symbiotic nitrogen fixation.

The presence of bound γ-aminobutyric acid in such large amounts in white clover nodules and in smaller amounts in nodules of *T. pratense* and *T. medium* is therefore of particular interest. It would appear from Table 1 that the occurrence of bound γ-aminobutyric acid in root nodules is likely to be confined to the genus *Trifolium*. The amounts of bound γ-aminobutyric acid observed in white clover nodules represent about 20 per cent. of the total nitrogen content. The smaller amounts present in nodules of *T. pratense* and *T. medium* are possibly related to the slower growth of these two species at the time of sampling. From sampling of white clover nodules at various times during the year from plants growing under field conditions, it appeared that the content of bound γ-aminobutyric acid was directly related to the rate of nitrogen fixation, as assessed by the vigour and rate of growth of the plants. The small amount of bound γ-aminobutyric acid observed in ineffective nodules of white clover is in accord with this observation.

In the white clover plant, bound γ-aminobutyric acid appears to be virtually absent from tissues other than those involved in active fixation of nitrogen—it may therefore be involved in nitrogen fixation processes in this species.

Chemically bound β-alanine and γ-aminobutyric acid were found by Virtanen and Miettinen (1953) in pea plants; they suggested that compounds of amino acids with sugars might be involved. The state of combination of γ-aminobutyric acid in our material remains a matter for further investigation.

## V. Acknowledgments

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# THE EFFECTS OF WASHING TREATMENTS ON THE COMPOSITION OF *SALMONELLA ORANIENBURG*

By J. H. B. Christian*

*Summary*

Cells of *Salmonella oranienburg* grown in liquid media of water activity ($a_w$) 0·993 were washed in water and in solutions of sucrose, glycerol, NaCl, KCl, NH$_4$Cl, and MgSO$_4$ at a range of $a_w$. Retention of sodium and potassium by the cells and loss of compounds absorbing in the ultraviolet were measured. At pH 7·5, cells retained most sodium after washing in water, while retention of potassium and ultraviolet-absorbing compounds was greatest after washing in isotonic sucrose and electrolyte solutions. Changes in the pH of washing solutions markedly affected retention of cell constituents. When washed in solutions of $a_w$ from 1·000 to 0·990, the contents of potassium and ultraviolet-absorbing compounds in the cells were a function of $a_w$ while the sodium content was largely dependent on the nature of the washing solute. These results are discussed and recommendations made concerning the choice of solutions for washing and suspending cells of this organism.

## I. Introduction

The preparation of microorganisms for experimental purposes frequently involves a washing procedure to free the cells from contamination by constituents of their previous environment. Washing is usually performed with water, buffers, or salt solutions. For washing other particulate fractions (e.g. mitochondria) sucrose solutions are generally preferred. The effects of these treatments on the composition of the cell have not been widely considered, especially with bacteria.

A previous investigation (Christian 1955) showed that the amount of potassium accumulated by respiring cells of *Salmonella oranienburg* was related to the water activity ($a_w$) of the medium but that the sodium content of the cells was not. The present communication concerns the ability of cells to retain their internal sodium and potassium during washing in solutions of electrolytes and non-electrolytes. For comparison, the leakage of some compounds absorbing in ultraviolet light has also been followed. The effects of the composition of the growth medium, the nature of the washing solute, $a_w$, and the pH of the washing solution have been investigated. Large differences have been found in their effects upon leakage of sodium, potassium, and compounds absorbing at 256 m$\mu$.

## II. Methods

The test organism was a strain of *S. oranienburg* whose water relations had been studied previously (Christian 1955). Cells were grown in brain heart broth, or nutrient broth plus KCl (both media 0·993 $a_w$) for 16 hr at 30°C and aerated by

shaking. The stationary phase cells were harvested by centrifugation, and suspensions prepared in the spent medium at concentrations of about 60 and 120 mg wet cells per ml for cells grown in nutrient broth and brain heart media respectively.

Replicate 5-ml samples were dispensed in 7-ml glass centrifuge tubes, centrifuged, drained, and the tube walls blotted dry. After weighing, various pellets were used in washing experiments, for dry weight estimations after 20 hr at 98°C, and for determination of intercellular space.

Intercellular space was determined with cells grown in brain heart broth by the method of Conway and Downey (1950), using sucrose as the test solute. A small known volume of sucrose solution ($0\cdot993$ $a_w$) was added to the centrifuge tube and the cells resuspended in it. After centrifugation the sucrose concentration in the supernatant was determined using the benzidine reagent of Jones and Pridham (1954). From the degree of dilution of sucrose by interstitial liquid an intercellular space of $2\cdot27\pm0\cdot21$ ml per g dry weight was calculated. A similar value is obtained from the data of Mitchell and Moyle (1956) on the phosphate-impermeable space in pellets of *Bacterium coli* at the same $a_w$. Experiments with *S. oranienburg* in which centrifugation was delayed until 1 hr after suspension showed slow penetration of the cells by sucrose. Hence the value found here is believed to be a reasonable estimate of the interstitial space.

Cells were washed in water and in solutions of sucrose, glycerol, NaCl, KCl, $NH_4Cl$, and $MgSO_4$ at $0\cdot993$ $a_w$ and in solutions of sucrose, $NH_4Cl$, and $MgSO_4$ at $a_w$ ranging from $1\cdot000$ to $0\cdot990$. 5-ml aliquots of the solutions were added to the wet pellets, the cells suspended with a capillary pipette, and the suspensions centrifuged at ambient temperature. The supernatants, suitably diluted, were retained for measurement of absorption at a wavelength of 256 m$\mu$ in a Beckman DU spectrophotometer. The centrifugates were treated with $0\cdot25$ ml 40 per cent. trichloroacetic acid, suspended in glass-distilled water, and centrifuged. The supernatants were analysed for sodium and potassium in an E.E.L. flame photometer. Sodium was not measured in NaCl-washed cells nor potassium in KCl-washed cells. The concentrations of ultraviolet-absorbing compounds in the washings were expressed as optical density per 100 mg dry wt. of cells, and the sodium and potassium in the cell pellets as $\mu$-equiv. per 100 mg dry wt. of cells.

The washing solutions were not buffered. The pH of the suspension during washing was controlled by the pH and buffering capacity of the cells and the interstitial growth medium. To regulate the pH during washing, the pH of the stock suspension was adjusted to the desired value with HCl. The pH of unadjusted suspensions from both media was $7\cdot5$–$8\cdot0$, and washing experiments were performed at pH $4\cdot5$, $6\cdot0$, and $7\cdot5$ with cells from brain heart broth.

## III. Results

### (a) Sodium and Potassium Content of Unwashed Cells

Unwashed cell pellets from brain heart broth contained $2\cdot27$ ml of intercellular medium per g dry wt. and the concentrations of sodium and potassium in this medium were 146 m-equiv./l and 24 m-equiv./l respectively. When the results of whole-

pellet analyses were corrected for these extracellular contributions the cells contained $15\cdot4$ $\mu$-equiv. sodium and $35\cdot4$ $\mu$-equiv. potassium per 100 mg dry wt.

On resuspending the cells in washing solutions, the interstitial medium was diluted by a factor of 20. If the same degree of packing is assumed in the pellet before and after washing, the contribution of the intercellular space of the washed pellet was $1\cdot6$ $\mu$-equiv. sodium and $0\cdot3$ $\mu$-equiv. potassium per 100 mg dry wt. Hence if there were no leakage of sodium or potassium from the cells during washing, the maximum recoverable value in the pellet would be 17 $\mu$-equiv. sodium and $35\cdot7$ $\mu$-equiv. potassium per 100 mg dry wt.

The internal water content of the cell was obtained by subtracting the water content of the intercellular medium from that of the whole pellet. On this basis it was calculated that the concentrations of sodium and potassium within the cell, assuming them to be free in the aqueous phase, were 94 and 216 m-equiv./l respectively.

(b) *Influence of the Washing Solute on Contents of Cells Grown in Brain Heart Broth*

Brain heart broth (37 g dry matter/l) contained sodium and potassium at concentrations of 146 and 24 m-equiv./l, a sodium : potassium ratio of $6\cdot08$. The yield of cells was about 2 g dry wt./l. Cells were washed in various solutions of $0\cdot993$ $a_w$ and in water ($1\cdot000$ $a_w$).

The amounts of sodium retained in the cell pellet after one washing at pH $7\cdot5$ in various solutions and in water are shown in Figure $1(a)$. The sodium values in glycerol- and in water-washed pellets of $17\cdot0$ and $18\cdot4$ $\mu$-equiv. per 100 mg dry wt. respectively are close enough to the calculated content of $17\cdot0$ $\mu$-equiv. to conclude that none of the sodium within the cell was lost during washing in either of these media at pH $7\cdot5$.

Figure $1(b)$ shows the effects of washing treatments at pH $7\cdot5$ on the potassium content of cell pellets. Under the best conditions the recovery of potassium after washing was about 31 $\mu$-equiv. per 100 mg dry wt. Since the theoretical content was $35\cdot7$ $\mu$-equiv., none of these treatments completely prevented the loss of intracellular potassium.

The ultraviolet absorption spectrum of supernatants from cells washed in water showed a marked maximum at 256 m$\mu$, and the absorption of all washings from these experiments was measured at this wavelength. Figure $1(c)$ shows values obtained after washing at pH $7\cdot5$. It is apparent that none of these treatments prevented the loss of these substances, presumably largely purines and pyrimidines, from the bacterial cell.

(c) *Influence of the Washing Solute on Contents of Cells Grown in Nutrient Broth plus KCl*

Nutrient broth, $0\cdot999$ $a_w$, was adjusted to $0\cdot993$ $a_w$ by addition of KCl. The medium then contained 10 m-equiv. sodium and 193 m-equiv. potassium/l, a sodium : potassium ratio of $0\cdot052$. This contrasts with the ratio of $6\cdot08$ for brain heart broth.

The yield of cells was about $0 \cdot 5$ g dry wt./l. Washing experiments were performed at pH $7 \cdot 5$ and the results are shown in Figure 2.

The trends were broadly similar to those observed with cells grown in brain hearth broth, but there were some differences. Relative to the other treatments, washing in $MgSO_4$ and glycerol solutions retained less sodium than with cells grown
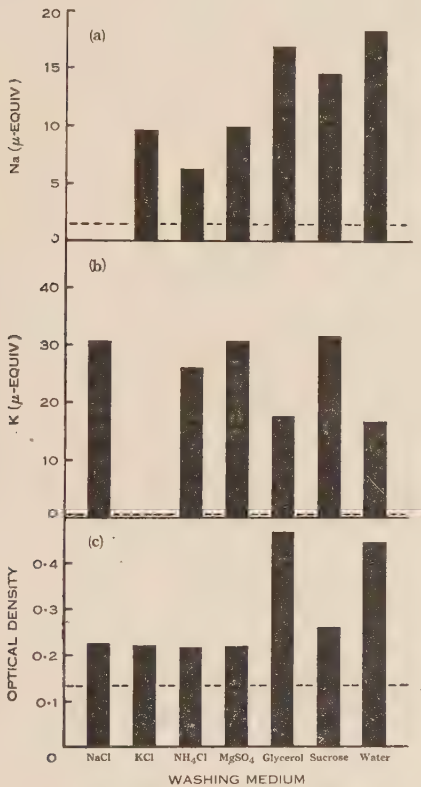


Fig. 1
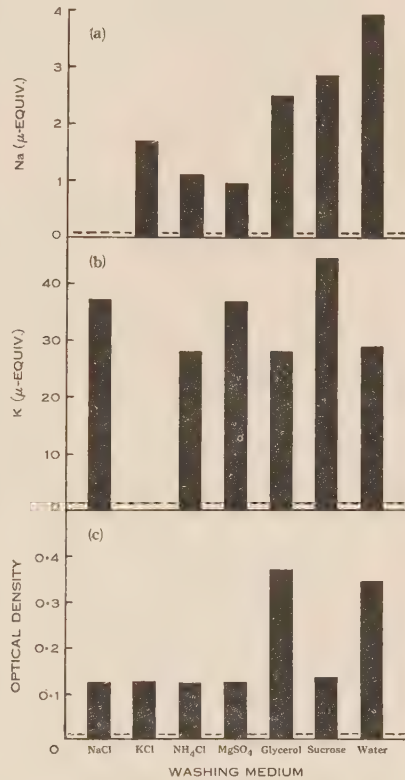
Fig. 2

Figs. 1 and 2.—Effect of washing at pH $7 \cdot 5$ in solutions of $0 \cdot 993$ $a_w$ and in water on the composition of *S. oranienburg*. Cells grown in brain heart broth ($0 \cdot 993$ $a_w$) (Fig. 1) and nutrient broth plus KCl ($0 \cdot 993$ $a_w$) (Fig. 2). Broken lines indicate the contribution of contaminating growth medium. (a) Sodium content of cell pellet. (b) Potassium content of cell pellet. (c) Optical density at 256 m$\mu$ of washings after dilution to one-tenth. Values are per 100 mg dry wt. of cells.

in brain heart broth (Fig. 2(a)). Sucrose was clearly superior to all other solutes tested in preventing leakage of potassium (Fig. 2(b)) while $NH_4Cl$ was no better than glycerol or water. Figure 2(c) indicates that the leakage of compounds absorbing in the ultraviolet followed the same pattern as was found for cells from brain heart broth.

## (d) Effect of pH

Cells grown in brain heart broth were washed in various solutions of $0.993\ a_w$ and in water at pH's of $4.5$, $6.0$, and $7.5$. Figure 3 shows that, in general, sodium retention was lowest at about pH $6.0$ and that differences between treatments were
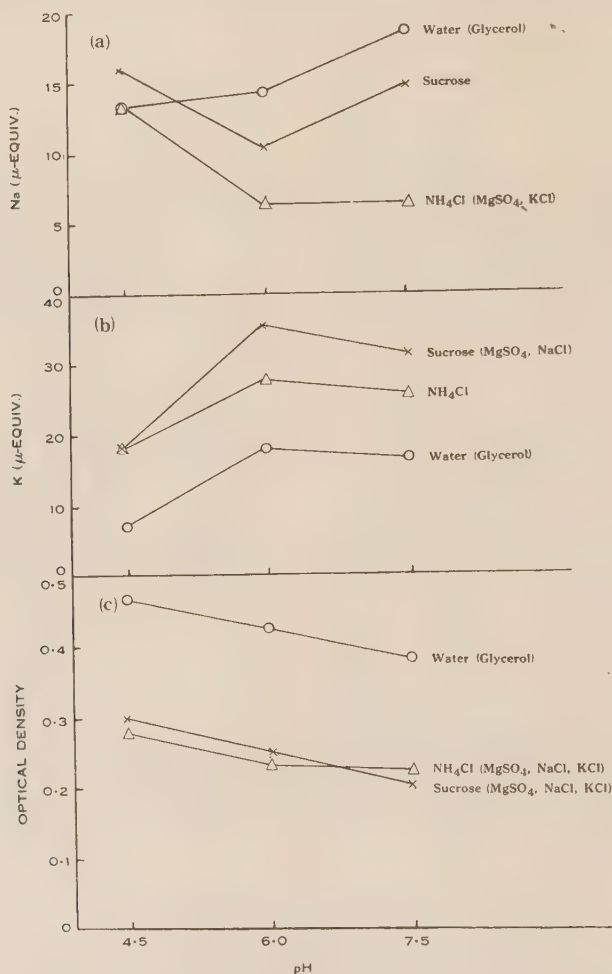


Fig. 3.—Effect of pH during washing in solutions of $0.993\ a_w$ and in water on composition of *S. oranienburg*. Cells were grown in brain heart broth ($0.993\ a_w$) and washed in sucrose ($\times$), $NH_4Cl$ ($\triangle$), and water ($\bigcirc$). Solutes in parenthesis gave results similar to the adjacent curves. (a), (b), and (c) as in Figures 1 and 2.

least at pH $4.5$. The potassium content of cells was greatest at pH $6.0$ and cells washed at this pH in all three solutions contained amounts of potassium within 5 per cent. of the corrected content of unwashed cells. Thus if unwashed cells in a medium of pH $6.0$ have the same potassium content as those at pH $7.5$, these three treatments at pH $6.0$ prevent its loss from the cells. Change in pH did not affect the relative

efficiencies of the three solutions in preventing loss of ultraviolet-absorbing compounds but losses increased as the pH fell from 7·5 to 4·5.

### (e) Effect of Repeated Washings

In experiments so far reported, cells were washed in the presence of medium carried over in the interstices of the cell pellet. To determine whether the results were affected by the small volume of solution used, cell pellets were washed three times in sucrose solutions or in water and analysed for sodium and potassium after each washing. The results are given in Figure 4.
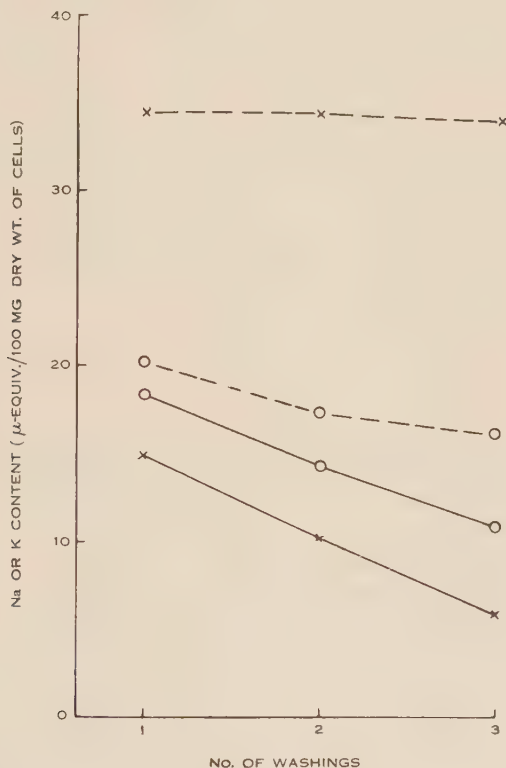


Fig. 4.—Effect of repeated washing at pH 7·5 in sucrose solution of 0·993 $a_w$ and in water on the sodium and potassium content of *S. oranienburg*. Cells grown in brain heart broth (0·993 $a_w$). ——Sodium content. – – –Potassium content. × Sucrose-washed cells. ○ Water-washed cells.

These data confirm the validity of experiments on potassium leakage, since very little potassium was lost in the second and third washings in either sucrose solution or water. When cells were washed once in sucrose and then once in water the amounts of sodium and potassium retained were consistent with the results of Figure 4. These facts suggest that the degree of dilution of the growth medium was not an important factor in potassium retention. The steep fall in sodium content during the second wash in water may have been due to dilution of the contaminating medium, or to the leakage of some other substance not estimated. The same factors

may affect the sodium status of sucrose-washed cells, but it remains true that cells retain much more sodium after repeated washings in water than after identical treatment in sucrose solutions.
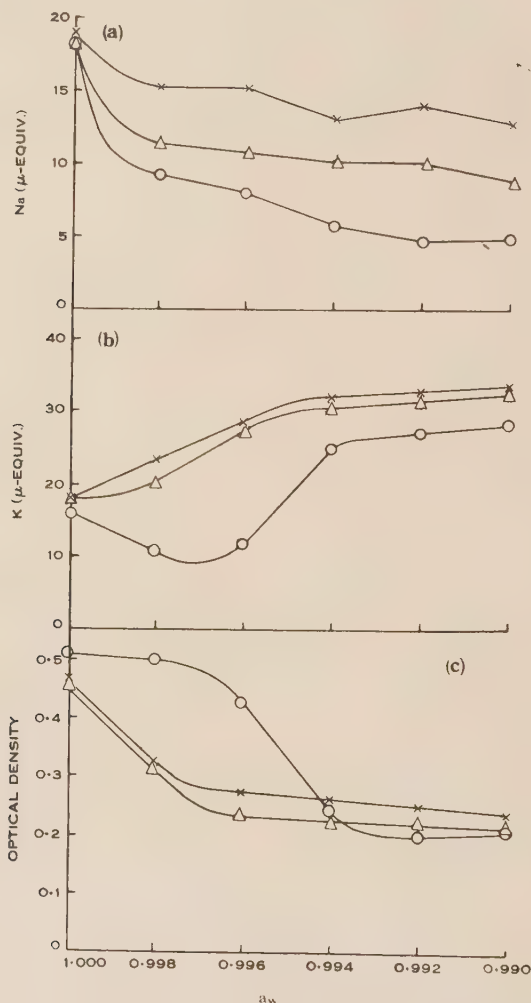


Fig. 5.—Effect of $a_w$ of the washing solution on composition of *S. oranienburg*. Cells were grown in brain heart broth (0·993 $a_w$) and washed at pH 7·5 in solutions of sucrose ($\times$), MgSO$_4$ ($\triangle$), and NH$_4$Cl ($\bigcirc$). (a), (b), and (c) as for Figures 1 and 2

## (f) Effect of a_w of the Washing Solution

In view of the large differences in the composition of cells after washing in water and in most solutions at 0·993 $a_w$, the effect of $a_w$ of the washing solution was studied. Cells grown in brain heart broth were washed in solutions of sucrose, NH$_4$Cl, and MgSO$_4$ at several $a_w$ between 1·000 and 0·990. Sucrose was chosen for its

overall efficiency in preventing leakage, $NH_4Cl$ for its inefficiency, and $MgSO_4$ for its similarity to NaCl and KCl in experiments at $0\cdot993$ $a_w$. The results are shown in Figure 5.

The differences in sodium content found previously for cells washed in the three solutions at $0\cdot993$ $a_w$ were largely maintained over the range $0\cdot990$–$0\cdot998$ $a_w$ (Fig. 5(a)). The differences were not eliminated until the $a_w$ was very close to $1\cdot000$, suggesting that retention of sodium by the cell depends much more on the nature of the solute in the suspending medium than on its concentration.

In all three washing solutions, gross leakage of potassium commenced when $a_w$ exceeded $0\cdot994$ (Fig. 5(b)). As with sodium, $NH_4Cl$ retained less potassium at all $a_w$ than the other solutions, but in this case the $a_w$ of the solution was more important than with sodium leakage.

The optical density of supernatants was not affected by hypertonic solutions, but increased rapidly when $a_w$ of washing solutions exceeded $0\cdot994$ for $NH_4Cl$ and $0\cdot997$ for sucrose and $MgSO_4$ (Fig. 5(c)). This points to another striking difference between the protection afforded by $NH_4Cl$ on the one hand and sucrose and $MgSO_4$ on the other, for while potassium and ultraviolet-absorbing compounds commenced to leak from cells at the same $a_w$ in $NH_4Cl$, they leaked at very different $a_w$ in the other two solutions.

## IV. Discussion

It is apparent from the results presented that none of the washing treatments tested is capable of retaining completely both the sodium and potassium content of cells of *S. oranienburg*. The similarity between the effects of water and isotonic glycerol solution on leakage of solutes is in accord with the well-known penetrating powers of glycerol and might have been predicted from earlier studies with this organism (Christian 1955). Here it was found that the effect of high concentrations of glycerol on respiration and potassium accumulation was negligible, while solutions of salts and sugars of the same $a_w$ were inhibitory.

Loss of sodium and potassium during washing with electrolytes is probably the result of an ionic replacement, but at $0\cdot993$ $a_w$ the effect is much larger with cellular sodium than potassium. The slight loss of both ions when cells are washed in sucrose is unlikely to be due to replacement by sucrose but rather to the presence of electrolytes carried over from the initial interstitial medium. The same carry-over is present in cells washed in water or glycerol which apparently lose no sodium. Thus a requirement for this type of replacement may be maintenance of a high effective osmotic pressure.

Since the differences in the effects of various solutes on leakage of electrolytes largely disappear when cells are washed at pH $4\cdot5$, it is probable that the pH becomes more important than the solute of the washing solution. However, the pH affects sodium and potassium leakage in opposite ways, and at low pH ($4\cdot5$) loss of potassium is at a maximum and of sodium at a minimum. This complementary response may be related to the linkage between sodium excretion and potassium accumulation which has been postulated for many other biological systems.

It has been claimed that cells of *Escherichia coli* are freely permeable to sodium chloride (Roberts *et al.* 1955). The sodium content of the cell might therefore be expected to fall rapidly on washing in water. Although it did fall steeply on the second and third washings, there was no loss in the first wash. During the first washing in water, the external sodium from brain heart medium is present at about 7·3 m-equiv./l. while the internal sodium concentration is about 94 m-equiv./l. The absence of leakage at a concentration gradient of 13 : 1 suggests that the cell does not allow free outward diffusion of the sodium which enters it during growth.

The two media on which cells were grown differed greatly in sodium and potassium content but were of the same $a_w$. Although no striking differences were observed in the response of the two types of cells to the washing treatments, the differences in their sodium and potassium contents deserve comment. The sodium content, after cells had been washed in water, was much higher in cells grown in the high-sodium medium. However, potassium contents, determined after sucrose washing, were little affected by the potassium content of the medium, viz. 31·6 and 44·5 m-equiv. potassium per 100 mg dry wt. for cells from media containing 24 and 193 m-equiv. potassium/l respectively. Hence the sodium content of cells is probably a function of the sodium content of the growth medium while the potassium content of cells is largely independent of the external potassium concentration. In the light of earlier experiments (Christian 1955) it is likely that the content of cellular potassium is largely a function of the $a_w$ of the growth medium.

The suggestion was made in this previous paper that the increase in potassium content of cells during respiration at low $a_w$ might be a major change by which the cell equilibrated to the more concentrated environment. Calculations made at that time based on internal water content equal to three times the dry weight showed that the lowering of internal $a_w$ by potassium, coupled with a univalent anion, would account for only two-thirds of the $a_w$ lowering of the external medium. Experiments have now shown that under these conditions of growth and centrifugation the internal water is only about twice the dry weight, and hence the internal concentration of potassium salts may be sufficient to balance the external $a_w$.

There is no evidence that all of the substances lost from the cells during washing were originally situated within the plasma membrane. With the compounds absorbing in the ultraviolet it is possible that the optical density found in excess of that due to carry-over from the growth medium was the result of desorbed purine and pyrimidine compounds from the cell surface. However, it seems likely, at least in the case of potassium and optical density measurements, that the compounds lost from cells in some hypotonic solutions were of intracellular origin.

Earlier experiments (Christian and Scott 1953) showed that the motile salmonellae are a homogeneous group in regard to their water relations. Thus it may be assumed that the results obtained with *S. oranienburg* apply to the whole genus and to the Enterobacteriaceae generally. However, some Gram-positive species behave very differently. Preliminary experiments with *Staphylococcus aureus* showed that little potassium was lost on washing in any of the isotonic solutions or in water, while with *Bacillus megaterium* the leakage of compounds absorbing in the ultraviolet was similar in all the solutions tested.

It is concluded that no one set of conditions tested completely prevented leakage of all the constituents studied from cells of *S. oranienburg*. However, the following suggestions may be made concerning the choice of solutions in which to wash or dilute suspensions of such cells: (1) concentrations hypotonic to the growth medium may be detrimental; (2) sucrose is probably preferable to electrolytes; (3) if electrolytes are used, those with cations of smaller hydrated ionic radii (e.g. potassium and ammonium) should be avoided; (4) a pH close to 7 is preferable to more acid conditions.

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# A STUDY OF VARIATION IN *NECTRIA STENOSPORA* BERK. & BR.

## By Ann Gibson* and D. M. Griffin*

*Summary*

This paper records a preliminary investigation of variation in *Nectria stenospora* Berk. & Br., a species which produces both sexual and asexual spores in culture. The cultural characteristics of the species and methods for isolation of single ascospores and conidia are described. Staining reveals that all cells of the frequently anastomosing mycelium are uninucleate. Two types of variation are described, one being a gene-controlled variation in colony colour, the other a cytoplasmically controlled variation in colony texture. Certain strains are shown to be self sterile, others self fertile although preferentially outbreeding.

## I. Introduction

Within the last few years the study of variation and genetics in fungi has revealed many features of great interest, such as heterokaryosis (Pontecorvo, Roper, and Forbes 1953; Pontecorvo *et al.* 1953; Buxton 1954), cytoplasmic variation (Jinks 1954; Arlett 1957), and the parasexual cycle (Buxton 1956; Pontecorvo 1956). This paper presents the results of a preliminary investigation of variation in *Nectria stenospora* Berk. & Br. (order Hypocreales) along with other notes on the species. The work has been briefly reported previously (Gibson and Griffin 1958).

## II. Materials and Methods

### (a) Vegetative Stage

*N. stenospora* is a common temperate and subtropical saprophyte, the present isolate having been obtained from the bark of a dying *Psoralia* bush at Sydney. The fungus grows on all the usual laboratory media but unless otherwise stated, all experiments were performed on 2 per cent. potato dextrose agar (P.D.A.) at 32°C at which temperature the initial rate of linear extension is *c.* 10 mm/day (7·5 mm/day at 25°C).

In order to study the assimilative hyphae without the interference of the aerial mycelium, a gently warmed slide was fused onto the surface of the agar so that it overlapped the edge of a vigorously growing colony. The hyphae continued to grow underneath the slide, forming normal assimilative mycelium. When the slide was prised off the agar, it came away bearing the uppermost layer of hyphae and could be passed through the staining process without any change in the orientation of the mycelium. The frequency of anastomosis in the assimilative hyphae was very great, the mature colony being in fact a complex mosaic of interconnected cells rather than an assemblage of radiating hyphae.

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These slide cultures, or conidia attached to a slide with albumen fixative, were stained by a modification of Heubschman's (1952) method. The material was fixed in fresh Carnoy's fluid for 30 min, washed, and immersed in 1N HCl at room temperature for about 12 hr, and subsequently stained for at least 30 min in a mixture of 50 ml 2 per cent. aqueous azur 1, 3 ml 10 per cent. $NaHSO_4$, and 3 ml 1N HCl. After washing in water, the slides were mounted in glycerine and examined. By this means it was shown that every cell, including the long cell at the tip of each hypha, where the nucleus was always found a little ahead of the last cross wall, contained only one nucleus. The only exceptions were a very few binucleate cells where it seemed reasonable from the position of the nuclei to postulate that mitosis had just occurred. These observations were confirmed by Giemsa staining. *N. stenospora* would appear to be unusual amongst members of the Ascomycetes and the Fungi Imperfecti in having consistently uninucleate cells.

### (b) *Imperfect Stage*

On the usual agar media, the fungus typically produces abundant conidiophores and wet phialospores best referred to the genus *Verticillium* Nees ex Wallr. (or to *Acrostalagmus* Corda if these are not considered synonyms). There is considerable variation, however, and some conidiophores resemble those of *Gliocladium* Corda whereas on sterile corncobs the conidia are borne in sporodochia referable to *Dendrodochium* Bon. or *Tubercularia* Tode ex Fr.

Staining with azur 1 revealed that the phialides were uninucleate as were the great majority of conidia, although a few of the largest contained two nuclei. The uninucleate nature of the phialides, however, insured the genetic uniformity of even these binucleate conidia. These observations are in accord with previous ones on fungi producing phialospores.

*Isolation of single conidia.*—Well-separated monoconidial colonies were obtained by streaking a suspension of conidia from slime balls over the surface of P.D.A. contained in a 10-cm petri dish.

### (c) *Perfect Stage*

In illuminated petri-dish cultures on P.D.A., certain isolates (see below) produced small, hard, brown structures near the edge of the dish. In cultures 9 or more weeks old, these structures had developed into mature, erumpent, ostiolate perithecia, orange in colour and with thin soft walls. They contained asci and typical two-celled hyaline ascospores, *c.* 10 by $3 \cdot 5 \mu$. In some cases the groups of perithecia were stipitate, being borne on the ends of stalks *c.* 3 mm long.

The earliest stage in perithecial formation observed was the occurrence of extensively coiled, robust hyphae which developed into hard knots of tissue and ultimately into perithecia. In the dark, or on Czapek-Dox agar, development did not proceed beyond the formation of the hard knots. Only coiled perithecial initials were developed under any conditions by infertile strains or crosses. Wheeler (1954) has described the blocking of the sexual process at various stages in *Glomerella cingulata*.

Although cytological studies were not made, it is presumed that, as in other fungi with binucleate ascospores, the two cells of the ascospore contain identical

nuclei, the final mitosis occurring after the delimitation of the spore and being followed by the laying down of the spore septum (Backus and Keitt 1940; Adam, quoted in Pontecorvo *et al.* 1953).

*Isolation of ascospores.*—The eight ascospores are obliquely uniseriate within the ascus until shortly before discharge. At full maturity the ascus becomes clavate and just protrudes through the minute ostiole, the spores now being clustered in a mucilaginous group at the top. The asci within a given perithecium discharge successively but the eight spores from each ascus are shot out in a group to adhere to any surface within a few centimetres. Thus well-separated groups of eight spores can be obtained on a surface held for a short time over a group of mature perithecia.

The small size of the ascospores of *N. stenospora*, combined with the mucilaginous matrix surrounding them, made the normal methods of isolation developed for *Neurospora crassa* unsuitable. The features of the sexual stage described above led to the development of an alternative method. Gelatine was sterilized by fumigation with propylene oxide (Snyder and Hansen 1947) before mixing with sterile water. Perithecia were suspended above plates of the very thick jelly and allowed to discharge at 10°C, the low temperature keeping the jelly firm and retarding spore germination. Groups of eight spores were cut out and the gelatine dissolved from them in warm sterile water, the suspension being spread over a P.D.A. plate. Plates developing eight colonies contained the separate spores from one ascus.

## III. Experimental

### (a) Variation in Colour

Analysis of the ascospores from asci I, II, and III showed that, in each case, there had been a 1 : 1 segregation for "yellow" and "pale" colony colour. Both strains invariably bred true on transfer, either by single conidia or mycelium, through many generations and the difference is assumed to be gene-controlled. The colour difference is most conveniently shown on P.D.A., there being no difference on Czapek-Dox agar unless ammonia is substituted for nitrate as the nitrogen source.

In petri-dish cultures, the colour was best seen on the reverse side, the colour being present in the submerged mycelium and the immediately adjacent agar. The conidia varied from yellow to orange in yellow cultures whereas in pale cultures the reverse side and the conidia were pale yellow to buff. However, the difference between conidial colour of the two types was insufficiently constant and distinct to allow classification on this character alone and cultures were always differentiated on colony colour. The difference was best shown when the cultures were grown in the light, for the pigment then tended to fade and the slight yellow colour of the pale colonies was destroyed, leaving the yellow colonies still quite strongly coloured.

Petri dishes containing agar were inoculated at the centre with mixed suspensions of pale and yellow conidia and the resultant colonies were initially yellow. Frequently, however, straight-sided pale sectors appeared at various stages during the growth of the colony. Such colonies were called "mixed" colonies and it might be considered that in them the two strains had grown independently, although closely intermingled, and that the presence of the pale strain had been masked by the local

diffusion of the yellow pigment from the other strain. Only if a considerable area of pure, or practically pure, pale strain occurred would its presence be revealed by a pale sector. However, the very abundant anastomoses between the hyphae make it impossible to assume that hyphae of different genotypes are physiologically independent of each other and it seems best to consider these mixed colonies as physiologically heterokaryotic. As the individual cells of *N. stenospora* are uninucleate, such a use would involve the widening of the concept of heterokaryosis and this is discussed further below.

TABLE 1

CHARACTERISTICS OF STRAINS DERIVED FROM ASCOSPORES OF ASCUS III

| Ascospore Strain* | Characteristics (see text) |
| --- | --- |
| 1, 2 | Self sterile, pale, uniform growth |
| 3, 4 | Self sterile, yellow, tasselled growth |
| 5, 6 | Self fertile, pale, ragged growth |
| 7, 8 | Self fertile, yellow, ragged growth |

*The numbering of the strains is solely for reference purposes and no order is implied for the parent ascospores.

### (b) Variation in Colony Texture

As shown in Table 1, the colonies derived from ascus III differed in their texture, being either "uniform", "ragged", or "tasselled". The character referred to as tasselled is not easy to describe, although very obvious visually (Plate 1, Fig. 1). On P.D.A., tasselled areas begin as variously shaped areas of the colony edge with reduced hyphal density and no conidiophores. From this sparse area, tassel- or fan-shaped areas of normal sporulating mycelium arise, grow for about 5 mm, and then in turn cease growth and give rise to a new tassel. The growth rate of such colonies is normal on P.D.A. Uniform and ragged colonies could only be distinguished in old, staled cultures (when ragged colonies had an uneven margin) and in most experiments they have been grouped together as being of normal appearance.

When subcultured by cutting out pieces of the agar and mycelium, all colonies maintained their characteristics unaltered through many transfers. In particular, the ragged areas at the edge of old colonies of strains 5–8 (Table 1) produced normal parental-type colonies on subculturing whereas the tasselled strains 3 and 4 maintained their abnormal growth form. Since only one pair of ascospores had given rise to tasselled strains it was possible that the character was controlled by mutant genes at two loci, e.g. $a$ and $b$. Thus the genotype $a^+b^-$ might be assigned to strains 1 and 2, $a^+b$ to 5 and 6, $ab^+$ to 7 and 8, $ab$ to 3 and 4. The hypothesis was tested in the following way.

(i) *Nature of Mixed Colonies.*—If the above hypothesis were true, it might be expected that the juxtaposition of nuclei bearing $a^+b$ and $ab^+$ would result in a

mixed colony of tasselled phenotype due to interactions through the anastomoses. Such colonies were therefore made by mixing conidial suspensions from strains 6 and 7 and plating them out and also by growing strains 6 and 7 adjacent to one another on the same plate so that anastomosis occurred at the junction. By both means tasselled growth was produced, in the first case at various areas throughout the colony after an initial period of normal growth (Plate 1, Fig. 2) and in the second case along a band marking the line of junction. Similar results were obtained by mixing strains 5 and 7 and, unexpectedly, in a few cases, by mixing strains 5 and 6. No tasselling was observed in mixtures of 1 and 6 but mixed colonies derived from strain 3 and any ascus III strain were uniformly tasselled.

The results are in accordance with expectation except for the production of tasselled areas in mixed colonies derived from the spore pair 5 and 6. This anomaly cast doubt on the accuracy of the initial hypothesis.

TABLE 2

CHARACTER OF MONOCONIDIAL DAUGHTER COLONIES FROM ASCUS III ASCOSPORE
STRAINS

| Ascospore Strain | Character of Monoconidial Daughter Colonies | | |
|---|---|---|---|
| | Normal | Tasselled | Tasselled (%) |
| 1 (uniform) | 48 | 0 | 0 |
| 1 (uniform) | 100 | 0 | 0 |
| 4 (tasselled) | 40 | 26 | 39·4 |
| 4 (tasselled) | 126 | 63 | 33·3 |
| 6 (ragged) | 41 | 6 | 12·8 |
| 6 (ragged) | 235 | 30 | 11·3 |

(ii) *Analysis of Colonies Developing from Monoconidial Isolates.*—Monoconidial isolates were made from many colonies derived either from single ascospores or from a mixed inoculum. Many monoascospore cultures were shown to produce monoconidial daughter colonies of two types and the results are shown in Tables 2 and 3.

Thus, although all these cultures maintained their characteristics if transferred by mycelium, only uniform strains produced wholly parental-type cultures if transferred by single conidia. Tasselled areas produced only 20–40 per cent. (mean = 31·7) tasselled colonies whereas normal (presumably ragged) areas produced 1–13 per cent. (mean = 9·7) tasselled colonies. The overall ratios for normal : tasselled daughter colonies were 304 : 141 and 409 : 44, for colonies derived from conidia from tasselled and normal (ragged) areas, respectively. These ratios are significantly different at the 0·1 per cent. level of probability ($\chi_1^2 = 66·2$).

(iii) *Proof of Cytoplasmic Inheritance.*—The results given above were strongly indicative that the tasselled character was cytoplasmically inherited and this was

proved by Jinks's (1954) test. Previously this test had only been applied to fungi which are morphologically heterokaryotic but there seems to be no reason why it should not be applied in the present case where, although morphological heterokaryosis was absent, there was ample opportunity for a given nucleus to become associated with the cytoplasm of another strain by virtue of anastomoses. A mixed colony was synthesized from conidia of strains 1 (pale, uniform) and 3 (yellow, tasselled), using colony colour as a nuclear marker. Single conidia from the yellow tasselled mixed colony so obtained were grown and some daughter colonies were both pale and tasselled. The tasselling effect had therefore become dissociated from the yellow-marked nucleus and associated with the pale nucleus. It is, of course, possible that the yellow nuclei had mutated to pale but it seems unlikely that the mutation rate is sufficiently high for this to be a likely explanation.

TABLE 3

CHARACTER OF MONOCONIDIAL DAUGHTER COLONIES FROM MIXED PARENTAL COLONIES

| Mixed Parental Colony and Area | Character of Monoconidial Daughter Colonies | | |
|---|---|---|---|
| | Normal | Tasselled | Tasselled (%) |
| 6 and 7 (normal area) | 84 | 1 | 1·2 |
| 6 and 7 (normal area) | 49 | 7 | 12·5 |
| 6 and 7 (tasselled area) | 62 | 29 | 31·9 |
| 6 and 7 (tasselled area) | 76 | 23 | 23·2 |

(iv) *Selection of Tasselled and Normal Strains.*—Jinks (1954) showed that the abundance of conidia in *Aspergillus nidulans* was controlled cytoplasmically and demonstrated the selection of an asexual strain by consistently subculturing with conidia. In the present experiment, two lines (normal and tasselled) were established from an ascospore 6 colony (ragged) and each line was then propagated for five asexual generations, in each case selecting for the line character. In each generation and line, approximately 50 conidia were taken from a single selected colony and analysed for the percentage of normal and tasselled strains.

It will be noted (Fig. 1) that the percentages in each line remain remarkably constant and after five generations there is no evidence of selection being effective. One conidium, however, from the first transfer gave rise to a normal colony which thereafter never produced tasselled cultures. This conidium had presumably been free of the tasselled factor.

(v) *Effect of Sexual Reproduction.*—From nearly all perithecia produced by strains from ascus III (see below), spores were formed which gave rise to tasselled colonies. One selfed perithecium from strain 6 produced only tasselled colonies from its spores.

### (c) *Variation in the Mating System*

Of the few asci analysed, ascus III was again the most interesting. As may be seen from Table 1, strains 1–4 proved to be self sterile, 5–8 to be self fertile. In contrast, no colony derived from a single ascospore from ascus I proved to be self fertile nor was any cross between the colonies fertile. In a previous paper (Gibson and Griffin 1958) the terms "homothallic" and "heterothallic" were used instead of "self fertile" and "self sterile". On the evidence available, however, it would seem preferable to use the latter terms.

Crosses between the pale, self-fertile strain 6 with either the yellow, self-fertile strain 7 or the yellow, self-sterile strain 3 were made. All crosses produced fertile perithecia and one ascus from each of several perithecia were analysed. Of the $6 \times 7$

ASCOSPORE 6 COLONY (RAGGED)

| GENERATION | NORMAL | TASSELLED | NORMAL | TASSELLED |
|---|---|---|---|---|
| | (%) | (%) | (%) | (%) |
| 1ST | 86·9 | | | 13·1 |
| 2ND | 85·7 | 14·3 | 66·0 | 34·0 |
| 3RD | 88·1 | 11·9 | 63·5 | 36·5 |
| 4TH | 87·5 | 12·5 | 70·7 | 29·3 |
| 5TH | 87·0 | 13·0 | 66·8 | 33·2 |
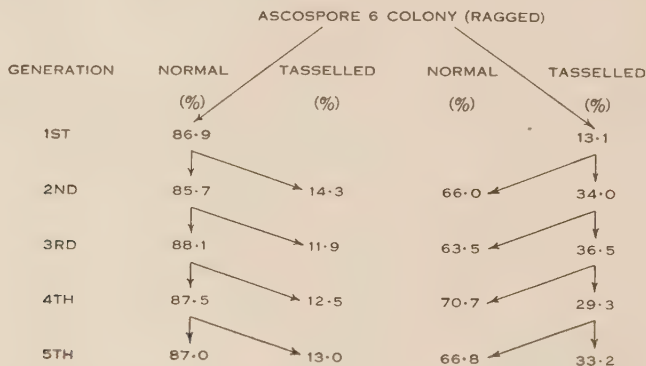
Fig. 1.—Analysis of five generations of monoconidial daughter colonies derived from an ascopore 6 colony.

crosses, three asci showed segregation for pale and yellow, a fourth ascus giving only pale progeny. Of the $6 \times 3$ crosses, both asci analysed showed segregation for the colour factor. Pure colonies of strain 6 gave only pale progeny from the four asci analysed. Therefore, although the number of asci analysed is admittedly very small, in five cases out of six the self-fertile strain 6 had outcrossed with either a self-fertile or a self-sterile strain. Such an effect has been described by Pontecorvo *et al.* (1953) for *Aspergillus nidulans* and there termed relative heterothallism. Such preferential outbreeding in homothallic fungi has also been described in *Sordaria fimicola* by Olive (1954) and in *Glomerella cingulata* by Wheeler (1954).

## IV. Discussion

The experiments described above suggest that it may be useful to widen the concept of heterokaryosis. The term "heterokaryotic" has generally been applied to a given cell and, by extension, to a colony composed of such cells, although Buxton (1954) has discussed the situation in *Fusarium oxysporum* in which only the cell at each hyphal tip is multinucleate. In the case of *N. stenospora*, individual cells are all uninucleate but the frequency of anastomosis is such that the colony resembles a cell mosaic. Thus although intracellular reactions between nuclei of different genotype

cannot occur, the many anastomoses provide a pathway for intercellular, interhyphal nuclear reactions. The yellow colony formed from the mixed yellow and pale conidia might therefore be considered heterokaryotic rather than consisting of a mixture of two strains with little connection except approximate concurrence.

Such a concept of heterokaryosis is supported by the work of Pittenger and Atwood (1956) working with *Neurospora crassa* heterokaryons formed from biochemically deficient strains. They have shown that the nuclear ratios in individual hyphae at the growth frontier varied widely and that the growth rate of the frontier depended upon the overall nuclear ratio of a large number of hyphae in the area. Thus the growth rate of hyphal tips did not reflect their genotype unless they were isolated from their neighbours. Such facts could only be explained on the assumption of interaction between the nuclei (or their products) from different cells and hyphae. The existence of cytoplasmic translocation between cells provides one means for such reactions.

Thus even with fungi with heterokaryotic cells, intercellular and interhyphal reactions can be the dominating feature. It seems not unreasonable to extend the term heterokaryotic to colonies of fungi with a mixed nuclear population, even if individual cells are uninucleate, so long as there are sufficient anastomoses to provide cytoplasmic continuity for interactions between the cells of different genotype even if they are in different hyphae, and the local colony character depends on the nature of the overall nuclear population in that area.

A second feature of interest is the variation in colony form in the cultures derived from ascus III, which is similar in some ways to that described by Stover (1956) in *Thielaviopsis basicola* in that two methods of subculturing, by single spores or mass transfers, result in colonies of different phenotypes. It seems clear that this variability is under cytoplasmic control. In only two strains (3, 4) derived from ascus III are the cytoplasmic particles controlling tasselling in sufficient quantity to permit expression of the effect in rapidly growing colonies, although they are present in four other strains (5–8). In these four strains the presence of the particles is only shown by the ragged edge of old colonies or by tasselling in daughter colonies derived from conidia which, by chance, incorporate a greater-than-average number of particles (Arlett 1957).

There, are, however, three points of note in connection with this cytoplasmically controlled variation. Firstly, the expression of the character is limited to those strains derived from ascus III. Thus although a mixed colony derived from strains 1 and 3 is tasselled, one between strain 3 and a strain from another ascus is normal. It would therefore seem either that the nucleus or the cytoplasm of these other strains suppresses the phenotypic effect of the particles even if they are present in sufficient numbers to cause its expression in combination with strain 1, itself completely or practically deficient in the particles.

Secondly, the distribution of the particles within ascus III must have been irregular, for one pair of spores had incorporated within them sufficient particles for phenotypic expression in their derivative colonies, two other pairs insufficient for expression, and one pair very few or no particles at all. That the number, or ratio, of particles may be altered after sexual reproduction is shown by the tasselled progeny from the perithecia of the self-fertile strain 6.

Finally, the percentage of normal and tasselled monoconidial daughter colonies produced in each generation remained unexpectedly constant, even under a selective pressure. There is, however, insufficient experimental evidence to postulate the cause.

Whilst further experiments will be needed for a better understanding of these problems, this investigation has revealed aspects of interest in the mechanism of variation in *N. stenospora*.

## V. Acknowledgments

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VARIATION IN NECTRIA STENOSPORA
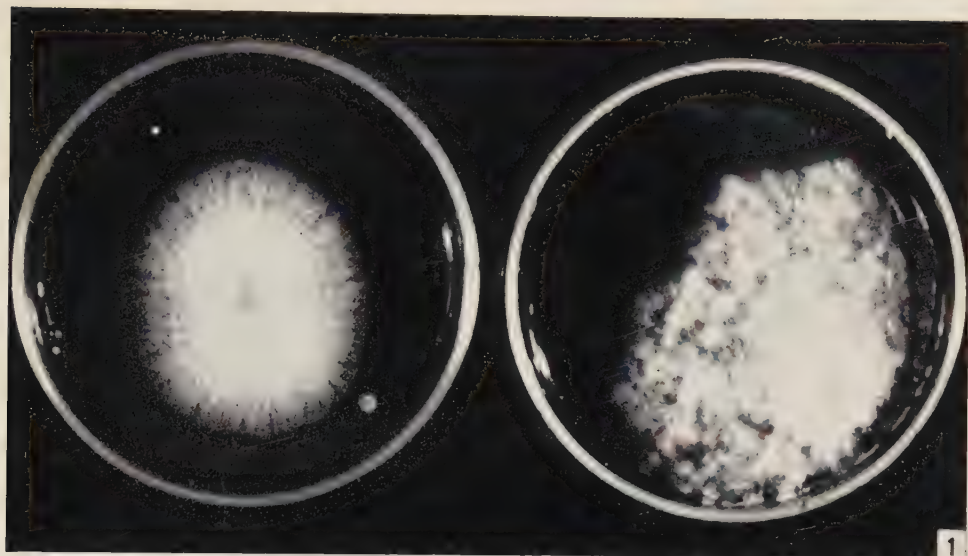


Fig. 1. –Colonies of *N. stenospora* of normal (left) and tasselled (right) morphology.   ×0·65

Fig. 2.—Tasselled areas occurring in a colony of *N. stenospora* after a period of normal growth, the colony originating from a mixed suspension of conidia from two ragged strains.   ×0·9.

# CELL CHANGES DURING PROTEIN SYNTHESIS AND SECRETION: AN ELECTRON MICROSCOPIC STUDY OF THE RESPONSE OF LYMPHOID TISSUE TO ANTIGENIC STIMULUS

By H. J. Hoffman* and G. W. Grigg*

### Summary

Mice were injected with doses of primary antigen (sheep erythrocytes or cholera vaccine) in the footpad. The majority received a second dose five days later. Lymph nodes were studied after preparation for electron microscopic examination at suitable times after the injection.

As early as 12 hr after a secondary injection, chromosomal filaments were denuded and displaced, shrinking to a fraction of their original thickness, presumably by shedding their histone sheath. The endoplasmic reticulum (cytoplasmic membrane) system underwent gross hypertrophy, and a substance resembling plasma proteins in appearance accumulated in its spaces. RNP granules were observed associated with the denuded chromosomal filaments and it is suggested that these filaments determined the specificities of the granules and also that the RNP granules migrated through openings in the nuclear membrane into the cytoplasm where they accumulated along the membranes of the endoplasmic reticulum. Later, the substance resembling the plasma protein in appearance, presumed to be the antibody protein, could not be seen in the spaces of the reticulum, and the reticulum was flattened. It is presumed that the antibody was extruded from the cells via the openings of the tubular reticulum in the cell wall.

A variety of lymphoid cells appear to undergo the changes described, which are considered to represent the secretory cycle initiated by antigen administration.

## I. Introduction

### (a) Nuclear Control of Protein Synthesis

Cytological changes associated with protein formation and secretion have been studied extensively in a variety of cell types. The investigations of Caspersson and his group (summarized in Caspersson 1950) based on quantitative histospectroscopy, and those of Brachet and collaborators (Brachet 1950) based on more conventional cytological methods both draw attention to the significance of the part played by the nucleus in regulating the cytoplasmic elaboration of proteins.

The "one-gene-one-enzyme" hypothesis (Beadle 1945) suggested a specific role of the gene in protein synthesis. Considerable data have since been collected to validate it as a general theory (cf. Mitchell and Lein 1948; Pauling *et al.* 1949; Fincham 1951; Giles, Partridge, and Nelson 1957). The simplest hypothesis relating

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the most constant element in the chromosome—deoxyribonucleic acid (DNA)—to the synthesis of specific proteins is that the DNA directly synthesizes protein; some instances of this are known (Allfrey 1954; Allfrey, Daly, and Mirsky 1955; Allfrey, Mirsky, and Stern 1955; Mirsky 1956). However, in most instances the DNA exerts a less direct influence (Gale and Folkes 1955; Brachet and Chantrenne 1956; Spiegelmann 1956) by way of the intermediary ribonucleic acid (RNA). The earlier correlations between this substance and protein synthesis were pointed out by Caspersson and Brachet. Caspersson (1950) has suggested that some of the cytoplasmic RNA, which he believed to be directly involved in synthesis of protein, is derived from the nucleus, the remainder being synthesized in the juxtanuclear zone of cytoplasm, under some regulatory control by the nucleus.

Experimental evidence has accumulated in support of the hypothesis that RNA is directly involved in protein synthesis. Slight reduction in the rate of RNA synthesis, achieved by use of inhibitors such as uracil analogues (Halversen and Jackson 1954) can profoundly affect rate of protein synthesis, while Allfrey, Daly, and Mirsky (1955) have demonstrated that ribonuclease (RNase) treatment of thymus lymphocytes will abolish amino acid incorporation into protein. Gale and Folkes (1955) have shown that in moderate depletion of the DNA and RNA of cell-free homogenates of bacteria, addition of RNA suffices to restore adaptive enzyme formation; in more severe depletion DNA must also be added. Spiegelmann (1956) has concluded from this and other evidence that RNA directly enters into protein synthesis, while DNA is involved less directly, by participating in RNA turnover.

Thus where cytoplasmic RNA is involved in protein synthesis, such synthesis is at least indirectly subject to nuclear control, by virtue of the role played by DNA. While "tracer" studies, such as the nuclear transplant experiments of Goldstein and Plaut (1955) support this nuclear origin, Brachet and Chantrenne (1956) have presented evidence in the alga *Acetabularia* that RNA can be synthesized in the cytoplasm. Moreover it is not certain that RNA of nuclear origin reaches the cytoplasm unchanged (Goldstein and Plaut 1955).

### (b) *Fine Structure of Cytoplasm and Nucleus*

With the electron microscope, extensive new information has been derived regarding the structure and chemical composition of the cytoplasm and the nucleus. The basophilic component of cytoplasm ("ergastoplasm") has been analysed by Porter (1953) and Sjostrand and Hanson (1954a). It appears to be composed of masses of granules 120–140 Å in diameter, extremely electron dense, which normally line lamellae, tubes, and spherical elements of the endoplasmic reticulum, and is an ubiquitous element of the cytoplasm first described by Porter, Claude, and Fullam (1945). In sites revealed by light microscopy to possess intense basophilia, as in ganglion cells (Causey and Hoffman 1955; Palade and Palay 1955) clumps of such granules are seen lying between elements of the reticulum. These granules, demonstrated by homogenization techniques in the microsome fraction by Littlefield *et al.* (1955), have been separated from the reticulum fragments, with which they occur in microsomes, by deoxycholate treatment (Palade and Siekewicz 1956) and

shown to consist of about 50 per cent. RNA and 50 per cent. protein. They are therefore called RNP granules in the description below.

Various workers have produced evidence of continuity between the plasma membrane and that of the endoplasmic reticulum (Rebhun 1956), and Palade (1956) has suggested this relationship is general. If so, the spaces of the endoplasmic reticulum must be continuous with the extracellular space.

Studies on nuclear fine structure have revealed (de Robertis 1956; Grigg and Hoffman, unpublished data) a major component composed of filaments, which coil to form the chromosomes of the mitotic phase. These filaments are of variable thickness, usually 50–70 Å, often spiralized in a helix of 150–250 Å diameter, and consist of a central core, of about 20–35 Å diameter, of DNA ensheathed in a variable amount of histone (Grigg and Hoffman, unpublished data). The thickness of the DNA core suggests that it is composed of one double helical molecule, if the Watson and Crick (1953) model is correct. The nucleolus, which is known to contain most of the nuclear RNA, seems to consist of chromosomal filaments bound up in an electron-dense amorphous material. RNP granules, as found in the cytoplasm, are not commonly seen in the nucleus although Porter (1954) and Gall (1956) have observed them there in some circumstances. As well as the histone (basic protein) of the chromosomal filaments, a second protein has been demonstrated. It lies between the filaments and accounts for a good deal of the electron density of the nucleus, and is not digested by trypsin. Papain removed it, and it has been tentatively identified as the acidic tryptophan-containing protein of the nucleus.

### (c) Antibody Formation in Lymphoid Tissue

As a model system for the study of cellular changes accompanying protein synthesis, the formation of antibody in lymphoid tissue in response to antigenic stimulus seemed eminently suitable to us, as the process is readily initiated and controlled.

It is generally accepted (McMaster and Hudack 1935; Ehrich and Harris 1942; Fagraeus 1948) that lymphoid tissue constitutes the principal site of antibody formation. The cell types responsible are still subject to controversy: Sabin (1939) considered that they were lymphoid stem cells, while numerous authors (summarized by Fagraeus 1948) correlate antibody titre with numbers of plasma cells, suggesting these latter are associated with synthesis. Harris *et al.* (1945) seems to have established convincingly the presence of antibody in lymphocytes; after a period of incubation in lymph this antibody passes out of the cells. Fagraeus's views do not conflict with these latter observations: she considers that the active cells may be of the lymphoid series, but that they transform into plasma cells. The only cells specifically excluded from antibody formation are the phagocytic cells.

A preliminary electron microscopic study of tissues containing numerous plasma cells (Braunsteiner and Pakesch 1955) indicates a characteristic appearance: this consists of greatly distended endoplasmic reticulum, with an appropriate increase in cytoplasmic volume, in comparison with the lymphocytic series.

## II. Material and Methods

Aliquots (0·02 ml) of the antigen used were injected into the left footpad of mice and the popliteal lymph nodes examined after appropriate periods. In some cases the next lymph node distal—the sublumbar—was also examined.

Three series of animals were examined: in the first two the antigen used was a 10 per cent. suspension of washed sheep erythrocytes. In the first series in which 14 animals were used, only a single injection was made, the animals being killed from 2 to 14 days after injection. In the second series of 27 animals a second dose of sheep erythrocyte antigen was given 5 days later. Animals were killed at periods from 12 hr to 7 days after the second injection. In a third series, cholera vaccine ($8 \times 10^9$ vibrio/ml, Commonwealth Serum Laboratories, Parkville, Vic.) was used as antigen in similar dosage and time relations, 11 animals being examined. Two animals with greatly enlarged axillary lymph nodes, resulting from low-grade chronic infection of a scabies lesion, provided further favourable experimental material while 10 untreated animals served as controls. At least two animals were used in each treatment group.

The animals were killed by a blow on the head and the popliteal lymph nodes rapidly exposed by splitting the fascia; chilled 1 per cent. osmium tetroxide solution, buffered at about pH 7·2–7·4 (Palade 1952) was then pipetted over it. The node was removed, divided into cubes of 1-mm side or less, and fixed in the osmium tetroxide solution at about $+4°C$ for 1 hr. The pieces were washed briefly in distilled water, dehydrated with absolute alcohol in an automatic discontinuous-feed dehydrator, then doubly imbedded, via alcohol–ether (1 : 1 v/v), in 4 per cent. celloidin under pressure, and finally blocked in 85 per cent. butyl and 15 per cent. methyl methacrylate (Grigg and Hoffman 1958). Sections were cut on a thermal-expansion microtome using a Fernandez Moran diamond knife (kindly presented to the authors by Professor H. Fernandez Moran). They were floated on distilled water, mounted on copper grids covered with "Formvar" films, the methacrylate removed after drying by dipping in carbon tetrachloride, and the sections in celloidin, viewed in the Phillips E.M. 100. Some were also viewed in the Siemen's U.M. 100 e.4. In some cases sections were stained by immersion in 0·1M ferric chloride solution for 5–15 min, then rinsed in distilled water and dried. This greatly increases the electron density of nucleic acid components (Bernstein 1956) especially of RNA. Sections from several regions of each block of tissue were examined with the electron microscope. This was repeated once or twice with other blocks from the same lymph node.

Sections 10 $\mu$ thick were cut from some of the plastic-imbedded tissues, mounted on albumen-treated slides, then treated with carbon tetrachloride and alcohol–ether (1 : 1 v/v) successively to remove methacrylate and celloidin. They were finally stained by the Unna-Pappenheim method to provide light microscopic controls on the electron microscopic investigations.

## III. Observations

### (a) Normal Lymphoid Tissue

Both fixed reticulum cells, and the free, rounded cells of the lymph node have been studied. Less attention has been paid to the small lymphocytes.

The general appearance and homogeneity of character of normal lymphoid cells can be seen in Plate 1, Figure 1, and Plate 2, Figure 2, which illustrate the appearance of groups of cells from normal lymph nodes. The nuclei were comparatively dense and packed with fibrils of about 50–100 Å thickness which were sometimes spiralized; their appearance in normal nuclei is indicated in Plate 1, Figure 1, inset. A diffuse material, removed by papain but not by trypsin (probably the tryptophan-containing protein (Grigg and Hoffman, unpublished data)) was observed to fill the space between the filaments.

The nucleoli were usually much more electron dense than the other regions in the nucleus, being composed of dense amorphous material (Plate 1, Fig. 1) superimposed on the chromosomal skeleton. As this plate illustrates, there was usually more than one nucleolus in each nucleus. Few, if any, granules similar to the RNP granules of the cytoplasmic reticulum could be observed in the nuclei of normal cells.

The nuclear membrane (Plate 1, Figure 1) normally had the characteristic two lamellae, and often, but not universally, was modified into the "pore" structure described by Afzelius (1955) and Watson (1955).

Cytoplasmic volume, composition, and elements varied from cell to cell, but, in general, the endoplasmic reticulum was found to be inconspicuous, composed of flattened crypts, readily visible in Plate 2, Figure 2, sometimes sparsely lined on the outer surface by RNP granules. However, as may be seen in both Plates 1 and 2, most of the RNP granules were clumped together with no obvious association with reticulum membranes. Granules and reticulum were both relatively sparse, whilst mitochondria, also sparse, were typical in appearance. Liposomes of characteristic structure (cf. Sjostrand and Hanson 1954b) were occasionally seen in normal cells.

### (b) Lymph Nodes of Experimental Animals

We shall discuss first lymph nodes from sequentially killed animals which had been treated with secondary antigen doses, largely because they present greater consistency in the changes observed, and enable a sequence of events to be described.

As early as 12 hr after the second injection of the antigen, changes were obvious in the nuclei of many cells—both reticulum cells and lymphocytes. Superficial examination of such nuclei (cf. Plate 3, Figure 5, and Plate 4, Figure 6) indicated loss of electron density in a patchy manner, regions becoming transparent; more detailed examination revealed (Plate 3, Figs. 3 and 5) that the filaments were altered both in distribution and character. In some regions filaments were absent, while they often appeared aggregated at the nuclear membrane. In other such regions (e.g. in Plate 3, Figs. 3 and 5; Plate 8, Fig. 17) the filaments appeared to be reduced to a fraction of their normal thickness and electron density. Measurement indicated that such denuded filaments had been reduced to a thickness of 20–30 Å, and in some instances one end of a filament was denuded while the remainder was still thicker and denser (cf. Plate 8, Fig. 17). The amorphous material between the filaments also seemed to disappear at about this stage, lending greater contrast to the fine chromosomal filaments. Comparison between apparently normal and denuded filaments, printed at the same magnification, is illustrated in Plate

1, Figure 1 (normal), and Plate 8, Figure 17 (denuded). Denuded filaments closely resembled normal nuclear filaments which had been digested with trypsin, while the disappearance of the amorphous material between the filaments was reminiscent of papain digestion (Grigg and Hoffman, unpublished data).

While these changes were occurring, the nuclear membrane became more conspicuous, due to marked increase in electron density (e.g. Plate 3, Fig. 5). In favourable situations it may be observed that this is not due to any basic alteration of lamellar structure, but to the presence of dense material on either side of the membrane.

Soon after filament denuding in the nucleus was first observed, lymph nodes were obtained in which the endoplasmic reticulum was extensively hypertrophied, producing an appearance such as in Plate 4, Figure 6, and Plate 5, Figures 9 and 10. The normally small flattened clefts were swollen, and gave the impression that many more diverticula of the reticulum system were formed. In some cases dense regularly arranged lamellar structures were present, similar to those described by Robertson (1954) and Palade and Palay (1955) which have been thought to signify zones of formation of new reticulum. Within the spaces of the reticulum moderately dense coagulated material was present, as seen in Plate 4, Figure 6, and Plate 5, Figures 9 and 10; in appearance it resembled the coagulated plasma proteins observed in capillary and lymphatic lumens.

These changes were amongst the most constant features observable in experimental lymph nodes from 12 to 72 hr after secondary antigen administration, and large groups of cells apparently underwent them synchronously. The electron micrographs shown in Plate 4, Figures 6 and 8, and Plate 5, Figure 9, all give the impression that at this stage the reticulum is tubular in nature, being wound around the nucleus. The response occurred with groups of cells rather than isolated individuals as illustrated by the group of three cells in Plate 4, Figure 7. All three are in the same state of hypertrophied reticulum, and it will be noted that in two of these cells the reticulum is oriented in a perinuclear, longitudinal fashion.

The description and interpretations of the nature of the endoplasmic reticulum mentioned earlier led us to search for continuity between cell membrane and reticulum membranes, i.e. openings of the tubes to the exterior. Several such openings were discovered, such as those seen in Plate 5, Figure 11, and Plate 6, Figure 12. In the latter figure, a connection between the cell membrane and the endoplasmic reticulum can clearly be observed, and within the open tube coagulated material is seen. In Plate 5, Figure 11, a number of diverticula of the reticulum may be seen in very close association with an invagination from the cell surface, suggesting that the section is close to the opening of the complex tubular system to the surface.

At the stage of maximal hypertrophy, the distended reticulum was generally sparsely lined with RNP granules; meanwhile, however, granules of similar size, shape, electron density, and staining character appeared within the nucleus. These granules were first observed on or in close proximity to denuded chromosomal filaments, as is seen in Plate 6, Figure 13, and Plate 8, Figure 17. These granules

first became conspicuous between 2 and 3 days after the second administration of antigen. In animals killed later than this greater numbers of granules were observed within the nucleus, always associated with the filaments, while numerous granules were to be observed in the nucleolei, as seen in Plate 6, Figure 14. Later, numbers of granules were observed under the nuclear membrane (see Plate 6, Figs. 13 and 14; Plate 7, Fig. 15) where often they tended to accumulate. The nuclear membrane often appeared modified in the regions of granule accumulation: in Plate 7, Figure 15, and its inset, tubular openings of diameter 200–300 Å are shown. Similar tubular openings were seen in nuclei where there were not such accumulations of granules, e.g. Plate 3, Figure 3, but the larger gaps in the nuclear membrane, as seen in both Plate 7, Figure 15, and Plate 8, Figure 16, were observed only where large numbers of granules were found. In the latter figure, one large and several smaller gaps in the nuclear membrane may be observed, with dense material including numerous granules in them.

The reticulum of many of the lymphocytes of animals killed 24 hr after those which had shown a grossly distended reticulum now appeared to consist of an extensive system of flattened clefts, as in Plate 4, Figure 8. When the reticulum diverticula appeared flattened, the relative volume of cytoplasm decreased, but the membranous system was still much more conspicuous than in normal lymphocytes, as seen in Plate 4, Figure 8. At a later stage the reticulum membranes again appeared inconspicuous while greater accumulations of cytoplasmic granules were observed. These were often present in a concentration far in excess of that in normal cells. Such a situation is illustrated in Plate 8, Figure 18, where the cytoplasm is now packed with clumps of granules, somewhat resembling the Nissl granules of nerve cells.

By this stage the nuclei were returning to their normal appearance; the chromosomal filaments were restored to their normal thickness of 50–100 Å. This restorative process in the nucleus was rather variable, however, the chromosomal filaments of some cells remaining denuded for long periods even after the cytoplasm had returned to normal. Thus the variability in restoration of the nucleus markedly contrasts with the uniformity of similar restorative processes in the cytoplasm.

During the period of active change in the composition and distribution of nuclear filaments, the nucleoli also underwent characteristic changes. Substantial increase in numbers of nucleoli and in their size and electron density was observed. These changes are clearly illustrated in Plate 4, Figure 6, where at least four nucleolar masses may be observed in one nucleus. Another feature typifying the changes occurring is the migration of two of these nucleoli to the nuclear membrane; this is characteristic of many cell types, e.g. the chromatolytic nerve cell regularly shows this type of nucleolar migration.

Examination of material 4 days after secondary antigen administration, in thick ($10\mu$) sections stained by the Unna-Pappenheim method, revealed numerous plasma cells and also large lymphocytes with large amounts of cytoplasmic RNA, supporting the correlation between plasma cells in light microscopy, and cells with distended reticulum, observed by Braunsteiner and Pakesch (1955).

In lymph nodes from animals receiving only primary antigen injection cellular response could be detected only several days after antigen administration; furthermore, no large groups of cells undergoing synchronous changes could be observed. Rather, cells in apposition were observed to be at different stages of the stages described above. Similarly, in lymph nodes from two animals with chronic low-grade cutaneous lesions, a wide scatter of stages could be detected. In this instance, however, a larger proportion of the cells differed from normal, and many cells were observed to be in the end-stage of intensely granular cytoplasm.

## IV. DISCUSSION

The earliest cellular changes associated with response to antigenic stimulus are the denuding of the chromosomal filaments, giving an appearance similar to that of filaments treated with proteases to remove the protein sheath, leaving only the DNA cores (Grigg and Hoffman, unpublished data) and the disappearance of the interchromosomal protein which we have considered to be the acid protein. Whether these changes accompany protein synthesis in all cases is not known; nuclei of secretory cells, such as the exocrine elements in the pancreas frequently present a similar appearance. It is possible that a small amount of such denuding accompanying RNP granule formation and protein synthesis occurs constantly in normal nuclei, and would be undetectable. These observations on histone movement and depletion in the nucleus are in general agreement with those of Caspersson and co-workers (Caspersson 1950).

The characteristic changes in the appearance of the nuclear membrane, concurrent with filament depletion, are suggestive of an accumulation of amorphous dense material on either side of the membrane. It is not unlikely that the diffuse density observed on either side of the membrane represents one or both of the proteins which are lost from the nucleus. Both Sjostrand (1953) and Palay (1956) have demonstrated similar changes in appearance of membranes of neural processes near the synaptic region: Sjostrand has attributed this to accumulation of dense amorphous material at the membrane interfaces.

The DNA skeleton of the chromosome appears to remain intact, and it seems possible that removal of the whole or greater part of the histone might facilitate the action of the DNA in forming RNA. Although RNA might be formed at a specific site and migrate to the chromosomal filament, there to be integrated with basic protein to form the typical RNP granule, it is not unlikely that it is formed by DNA, by a mechanism similar to that whereby DNA is thought to replicate itself (Taylor, Woods, and Hughes 1957). Since RNP granules increase in the nucleus and later in the cytoplasm and finally disappear in the nucleus, it appears to us that the RNA in the form of these RNP granules moves from its site of synthesis on the chromosome to the endoplasmic reticulum in the cytoplasm in an intact state. This is supported by biochemical evidence of Osawa, Takata, and Hotta (1957). Although Brachet and Chantrenne (1956) have queried the general occurrence of transfer of RNA formed in the nucleus to the cytoplasm, our evidence is consistent with such transfer. This does not suggest that all cytoplasmic RNA is formed in the nucleus, nor that all nuclear RNA is destined for the cytoplasm.

Further speculation as to the fate of the chromosomal histone, and the source of the basic protein which forms about half of the mass of the granules, might prompt the suggestion that the histone is incorporated in the granules; thus histone turnover might be part of the normal nuclear metabolism. RNA might well be in a similar constant state of synthesis and release in the chromosomes; the fact that small quantities may be identified normally in the chromosomes might support this. However, these matters cannot be determined until the basic protein of the granules has been more completely characterized.

That the granules observed in the nucleus are essentially similar to those normally occurring in the cytoplasm in association with reticulum is indicated by their similarity in size, appearance, and electron density and they stain similarly with Bernstein's ferric chloride technique. Further, some digestion experiments with RNase have indicated an essentially similar structure: in all cases the granules seem to possess an RNA core, with protein surrounding it (Grigg and Hoffman, unpublished data).

The detection of RNP granules in the nucleus only at certain stages of metabolic activity suggests that the nucleus may contain RNA organized in two physical forms, one associated with basic protein in granules, the other non-particulate and found concentrated in the nucleous of the quiescent nucleus. Nuclear RNA has been separated into two fractions on the basis of physical and chemical characteristics (Logan and Davidson 1957). Although RNP granules cannot be detected in numbers in the nucleus until after protein formation has ceased, it does not follow that granules are not synthesized actively at an earlier stage. If the RNA of the granule is inactivated and degraded during its activity in protein synthesis, and if the degradation rate equalled the synthetic rate, granules would not accumulate. Following cessation of protein formation, granules might then accumulate, until some simple "mass action" process inhibited this synthesis; meanwhile an accumulation of granules such as has been described would result. Since granule formation continued after the osmophilic material (presumably the antibody protein) disappeared from the interstices of the endoplasmic reticulum, availability of the granules in the cytoplasm is not the factor limiting the protein synthesis.

Modification of the nuclear membrane to permit the outward passage of the RNP granules is of some interest, although only the appearance of the larger gaps can be considered a specific response to the experimental procedure; tubes and pores can be observed in normal cells. Large gaps of the type described above have also been seen in cells forming intranuclear virus (Morgan et al. 1954; Harford et al. 1956). However, an essential difference is that the cell changes described here are reversible, since no gaps are observed when granule synthesis slows down. Extrusion of basophilic material in substantial quantities through the nuclear wall has been described in earlier literature (Goldschmidt 1910).

The substantial rise in cytoplasmic (and nuclear) ribonucleoprotein, demonstrated qualitatively in so many cytological investigations of antibody formation, and illustrated here by the large aggregation of granules, finds quantitative support in the studies of Ehrich, Drabkin, and Forman (1949), who showed a substantial rise in lymph node RNA in the period of 4–6 days after antigen administration.

The enlargement of the endoplasmic reticulum, and the extensive accumulation of osmophilic material which we assume to be the antibody being secreted into its spaces, with RNP granules collecting on the opposite side of the membrane, are all reconcilable with the general interpretations of the reticulum described in the introduction. This hypothesis, that the reticulum is an extensive complex invagination of the cell membrane is further supported by our finding "holes" in the cell membrane where the invagination occurs. Thus, throughout its life, the granule remains intracellular, while the secretion accumulates in what is an extension of the extracellular space. Since it is generally held that the enzymes associated with protein synthesis are either in the reticulum lipoprotein membranes or in the RNP granules, synthesis presumably occurs at the interface of granule and membrane, and in the 30 Å thick membrane itself. If Spiegelmann's (1956) view of the one-stage polymerization of protein is correct, the protein is formed almost at the extracellular site. Extrusion of the formed protein from the spaces of the reticulum which might result from contractile or amoeboid movements of the cell leaves the flattened spaces seen in Plate 5, Figure 9. It is significant that expulsion of secretory material from these swollen cells proceeds at a time when antibody titre has been shown to rise in similar experiments (Leduc, Coons, and Connolly 1955; Sobey, Adams, and Claringbold 1956).

Our tentative model for the synthesis of such specific proteins as antibodies provides for the transfer of information from the nucleus, where it is stored by the chromosomal DNA, to the site of cytoplasmic synthesis of the proteins by an intermediary molecule RNA. Spiegelmann suggests that for each specific protein to be formed, a specific RNA must be synthesized.

The information obtained in this investigation supports the view that the antibody synthesis occurring in the lymph node is carried on by a morphologically heterogeneous group of cells, ranging from reticulum cells to large lymphocytes. Thus the plasma cell loses its specific role and is confirmed as merely a lymphoid cell which has completed or is completing its secretory cycle, as suggested by Fagraeus (1948).

## V. Acknowledgments

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## EXPLANATION OF PLATES 1–8

### PLATE 1

Fig. 1.—Three cells from a lymph node of a normal mouse: the nuclei are moderately electron dense, the filaments $f$ being evenly distributed. In the lower nucleus several nucleoli ($n$) may be seen. These appear to consist of an amorphous dense material superimposed on a filamentous tangle. In the cytoplasm the elements of endoplasmic reticulum ($e. r$) are rare and mitochondria ($m$) appear typical, displaying cristae. Granules ($g$) of RNP are seen scattered in the cytoplasm in clumps. The nuclear membrane ($n. m$) and cell membranes ($c. m$) may be observed in contact.

*Inset.*—This represents an enlarged view of the region outlined in the lower nucleus: filaments may be seen at $f$.

### PLATE 2

Fig. 2.—Cells from normal lymphoid tissue. Nuclear filaments are readily seen, while the endoplasmic reticulum ($e. r$) is a little more prominent here, and appears to consist of small ovals. RNP granules ($g$) can be seen in small clumps; few are associated with reticulum.

### PLATE 3

Fig. 3.—Mouse lymph node 5 days after injection of secondary antigen (sheep red cells), showing portions of several large lymphocytes. In the cell on the left, the nucleus may be observed undergoing the characteristic denuding changes, denuded filaments being seen at $d$. The cytoplasm of this and the cells adjacent appears normal. Tubes ($t$) may be seen originating from the nuclear membrane and projecting into the nucleus.

Fig. 4.—A large lymphocyte from a node 8 days after secondary antigen administration. Nuclear filaments are still sparsely distributed, while four large nucleoli ($n$) may be observed, two of them apposed to the nuclear membrane.

Fig. 5.—A similar portion of the nucleus of a reticulum cell from a lymph node removed 48 hr after injection of secondary antigen. The fibrils are being dispersed, and some of the density of the interfibrillar spaces has been lost. Whilst many of the fibrils are still dense and typical, those at $d$ are becoming much thinner and fainter in appearance. Meanwhile, the nuclear membrane ($n. m$) in the upper portion of the field has become much more prominent: careful examination will reveal that the normal lamellae (at $n. m$) persist, but diffuse material is accumulating on either side of the membrane.

PLATE 4

Fig. 6.—Portion of a large lymphocyte from a node taken 48 hr after secondary antigen administration. Regions of the nucleus are severely depleted of filaments, the few filaments remaining in these regions being denuded ($d$). These may be compared with the thicker, denser filaments ($f$) of other regions. The endoplasmic reticulum ($e. r$) has increased tremendously in volume, and appears to contain amorphous material ($s$). The cell membrane can be seen at $c. m$, the small portion of cytoplasm of the neighbouring cell on the right shows similar reticulum hypertrophy. At this stage granules are largely absent from the reticulum.

Fig. 7.—Portions of three cells in an axillary lymph node adjacent to a chronic skin infection. All three cells show a similar degree of hypertrophy of the endoplasmic reticulum ($e. r$) which is seen to be sectioned longitudinally in the upper two cells. Note the concentric lamellae of the reticulum. Stained with ferric chloride (note ferric crystals at $Fe$).

Fig. 8.—Portion of a cell from an axillary lymph node of a chronic skin lesion showing substantial hypertrophy of the endoplasmic reticulum ($e. r$) with clearly observable material ($s$) in its spaces. In this instance the reticulum membranes appear to have been sectioned longitudinally showing the tubular nature of the system.

PLATE 5

Fig. 9.—A reticulum cell 3 days after administration of secondary antigen containing hypertrophic, but collapsed, flattened endoplasmic reticulum ($e. r$). It is assumed that this cell has expelled its secretion.

Fig. 10.—Concentric whorl of reticulum, from a cell sectioned eccentrically 3 days after administration of secondary antigen. The endoplasmic reticulum ($e. r$) is hypertrophic, and filled with an amorphous material ($s$).

Fig. 11.—A region of contact between two cells 4 days after administration of secondary antigen, showing how invagination of the plasma membrane ($c. m$) of the lower cell (arrows) forms a pocket apparently delimiting several diverticula of the endoplasmic reticulum ($e. r$). The situation is confused here, as the precise points of continuity between the elements do not appear to have been cut in this section.

PLATE 6

Fig. 12.—Portion of the cytoplasm of two adjoining cells 3 days after administration of secondary antigen. In the lower cell, at the point indicated by arrows, the plasma membrane ($c. m$) invaginates into the endoplasmic reticulum ($e. r$) which is hypertrophic and contains secretion.

Fig. 13.—Portion of a cell from a lymph node removed 5 days after antigen administration. Granules ($g$) may be seen within the nucleus, lying on chromosomal filaments ($f$). More granules are present under the nuclear membrane ($n. m$) while similar granules are seen in the cytoplasm. Sections stained by Bernstein's ferric chloride method.

Fig. 14.—A cell in similar conditions to that in the previous figure: granules ($g$) may again be seen accumulating on nuclear filaments, under the nuclear membrane, and in the cytoplasm. The intensely granular nature of the nucleolus ($n$) contrasts sharply with that of the quiescent cell. Granule contrast is enhanced by iron staining. From the same stage as Figure 13.

PLATE 7

Fig. 15.—Two cells from a lymph node draining a chronic skin lesion: the nuclei show evidence of filament depletion and denuding ($d$) and granules ($g$) can be seen on the filaments, and accumulating under the nuclear membranes (arrows). Tubular openings ($t$) in the nuclear membrane can be seen, and in places there are gaps ($ga$) marked by arrows through which granules are passing into the cytoplasm. The tubular openings are more clearly seen in the inset.

PLATE 8

Fig. 16.—Portions of the nucleus and cytoplasm of a cell 5 days after administration of secondary antigen showing large gaps (*ga*) in the nuclear membrane through which material, including granules (*g*), is escaping. Masses of similar material may be seen in the cytoplasm.

Fig. 17.—Denuded filaments (*d*), together with thicker filaments (*f*), in the nucleus of a large lymphocyte from a node removed 3 days after secondary antigen administration. In the filament indicated by arrows, portion has remained thick (*t. f*) while denuding has occurred in another portion (*d*). Granules (*g*) can be seen along this and other filaments. (Siemens U.M. 100 e.4.)

Fig. 18.—Portions of a group of cells from a node removed 12 hr after secondary antigen administration. The approximately trianular piece of cytoplasm on the left of the field retains some swollen reticulum, but has become extremely densely packed with granules (*g*) which are clumped together in groups. Granules can also be seen in the cytoplasm of the four cells adjoining it but in far lower concentrations, and also in all three of the nuclei in this figure.

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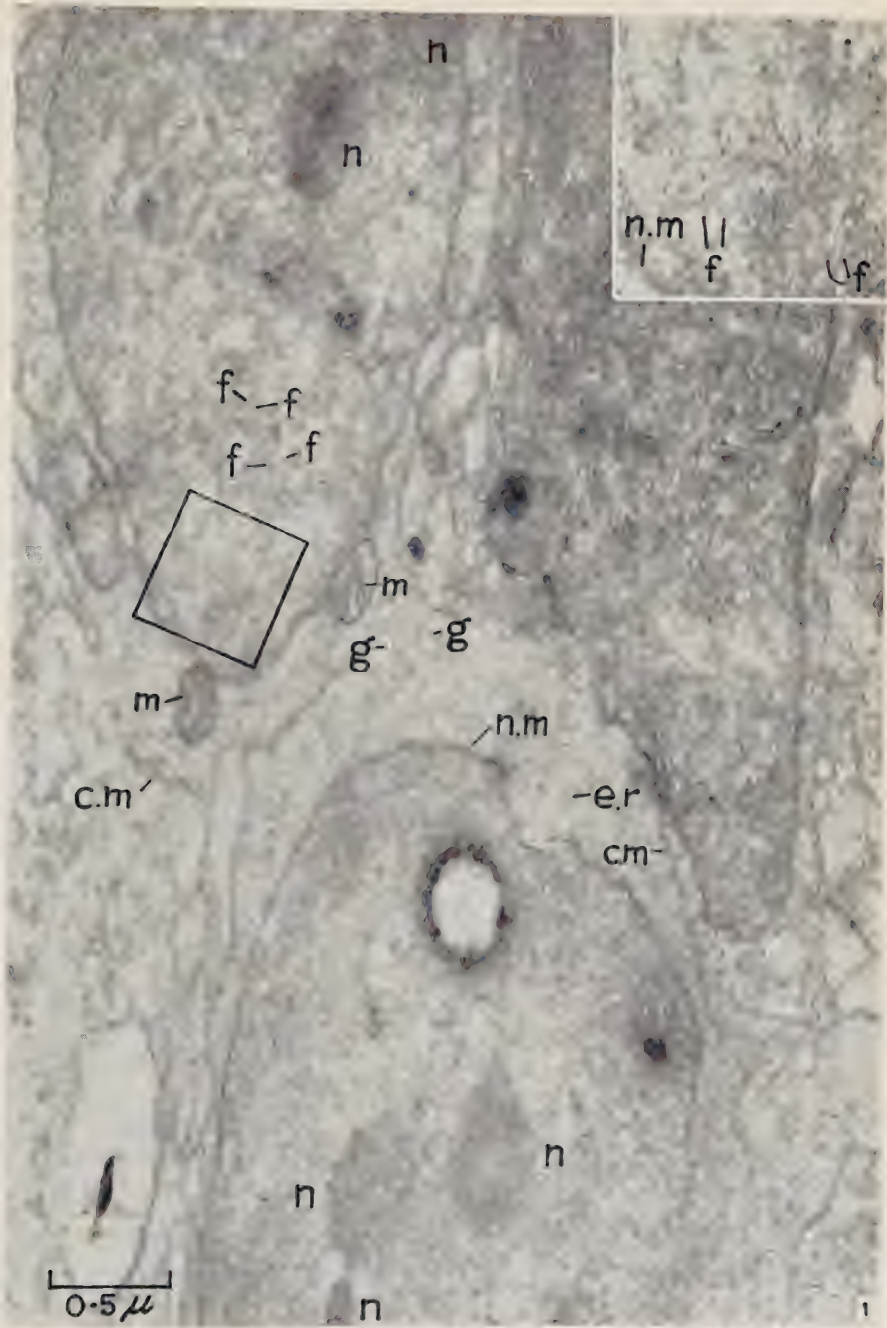
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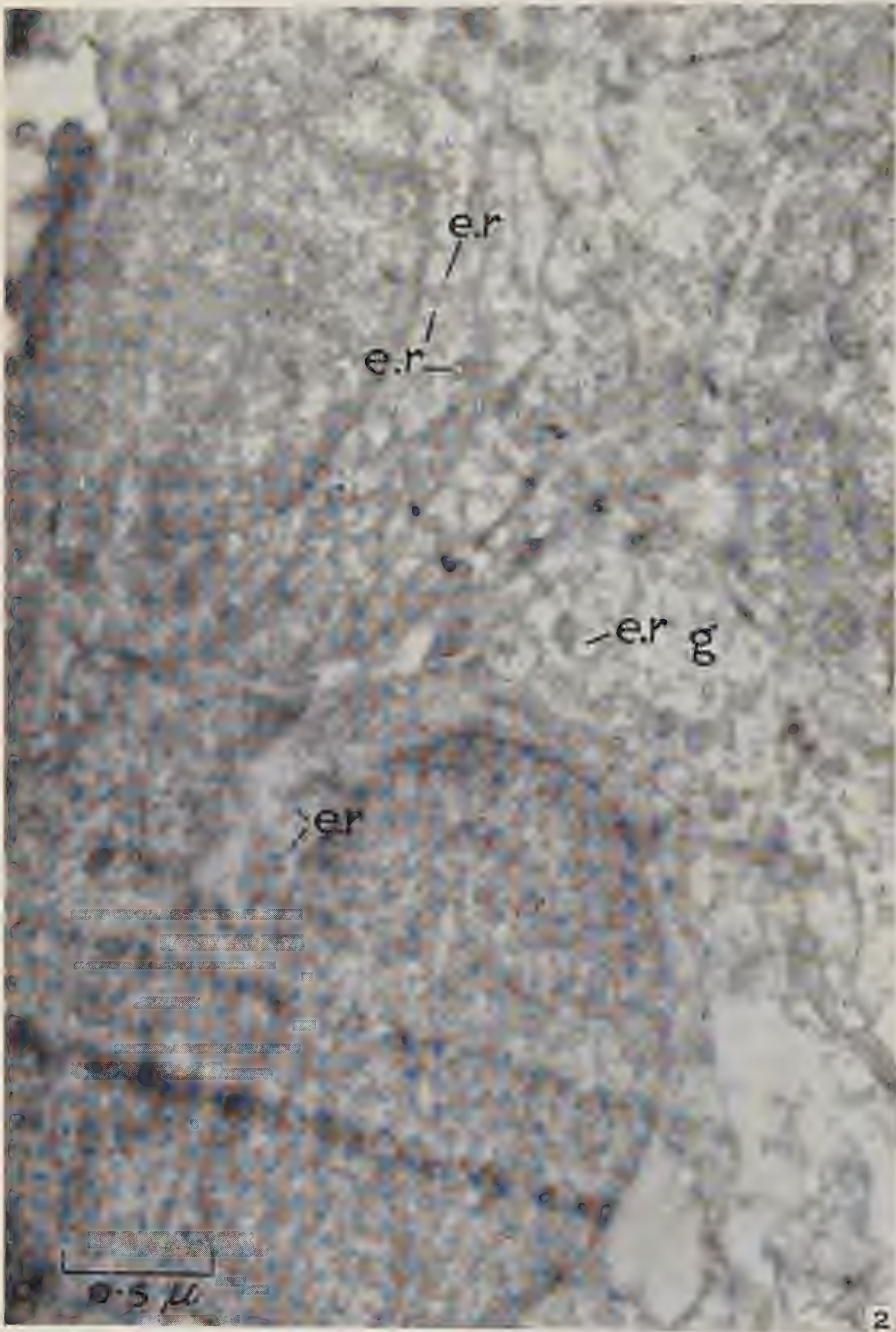
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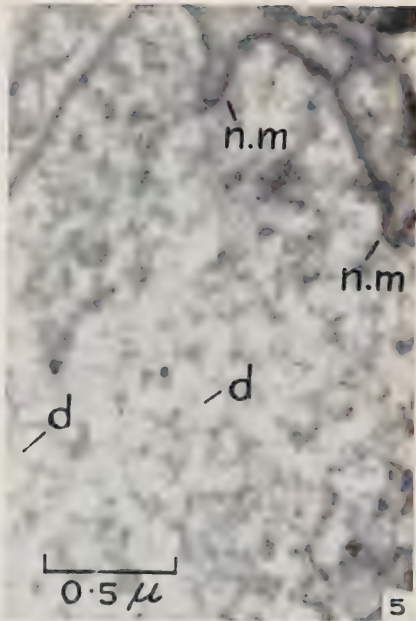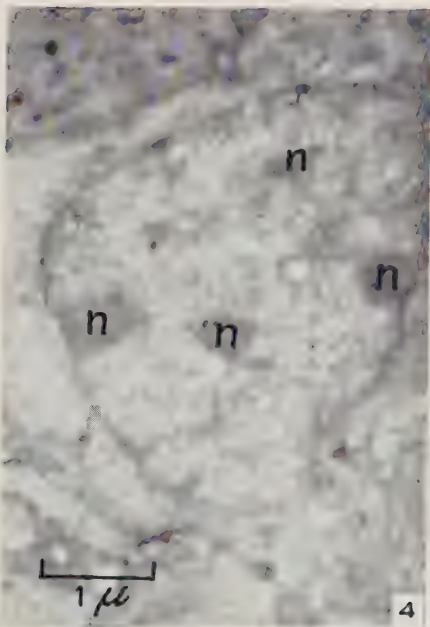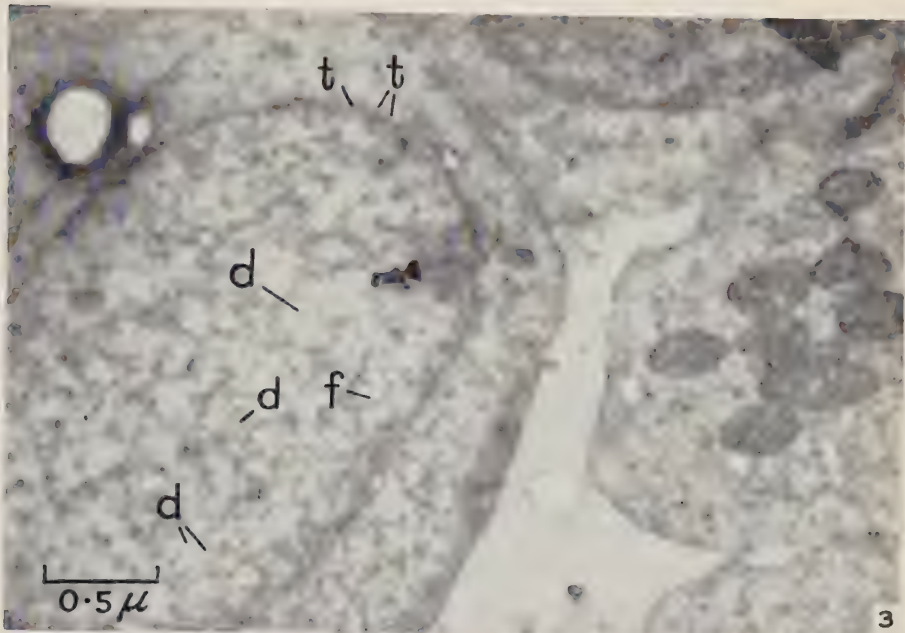
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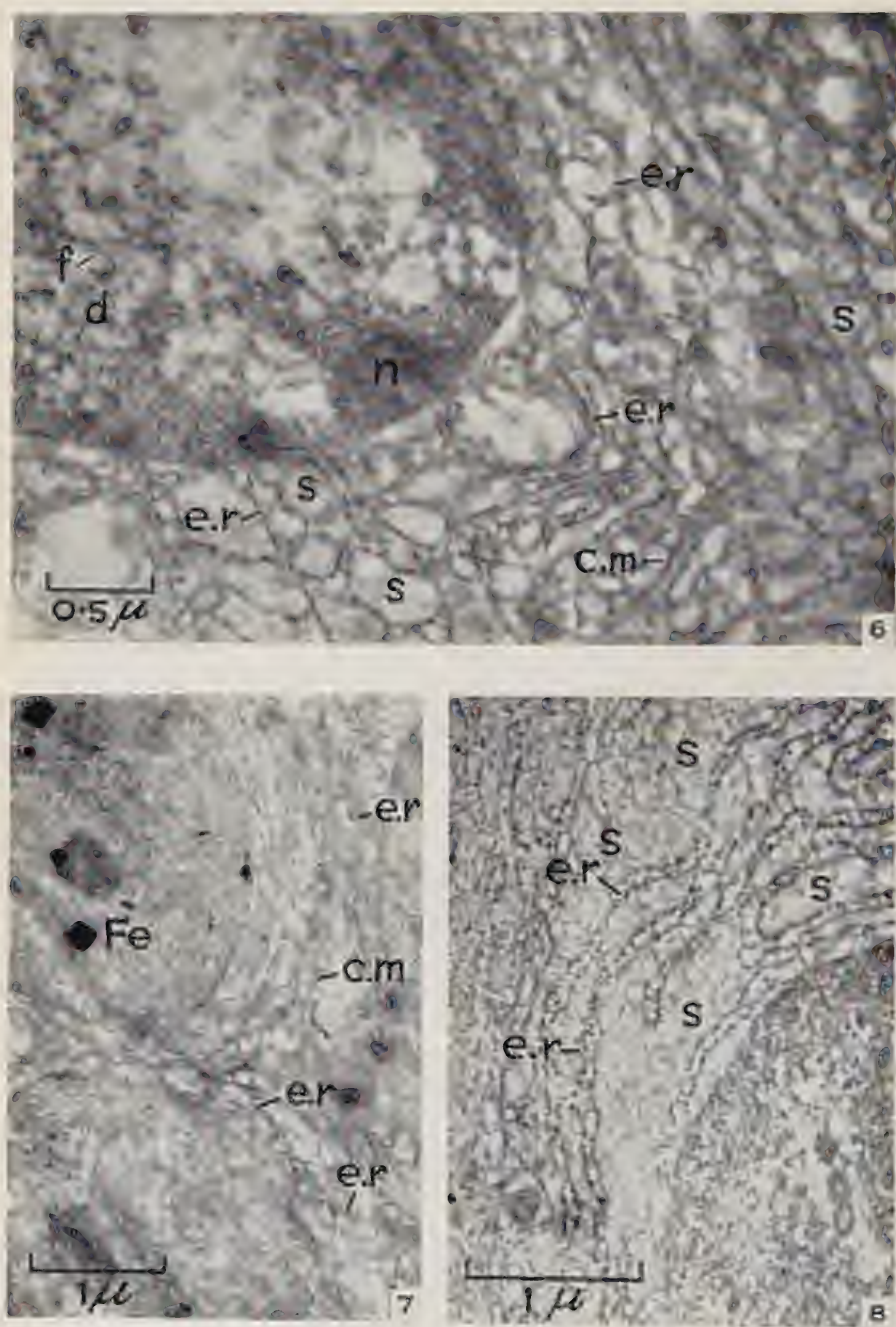
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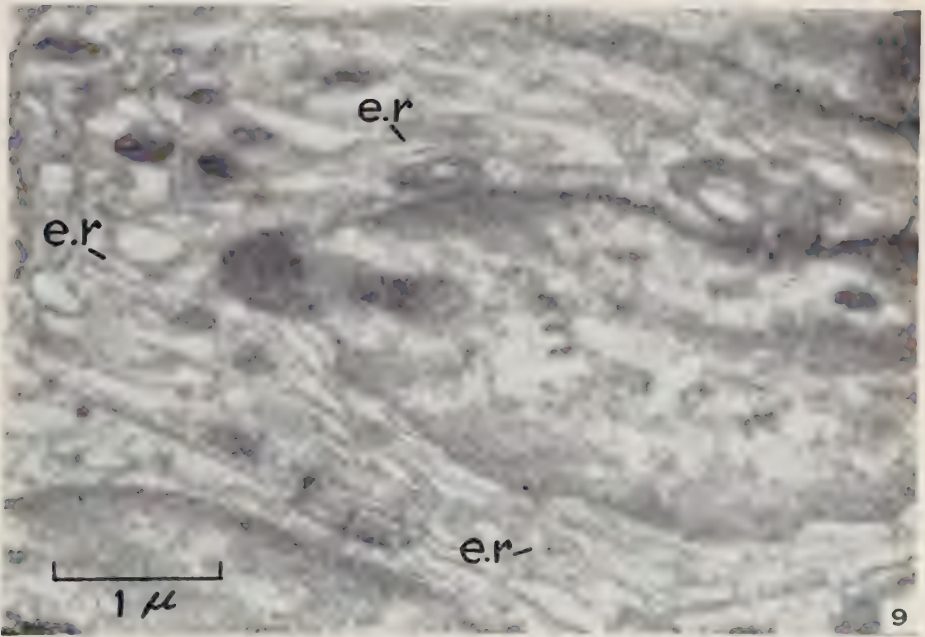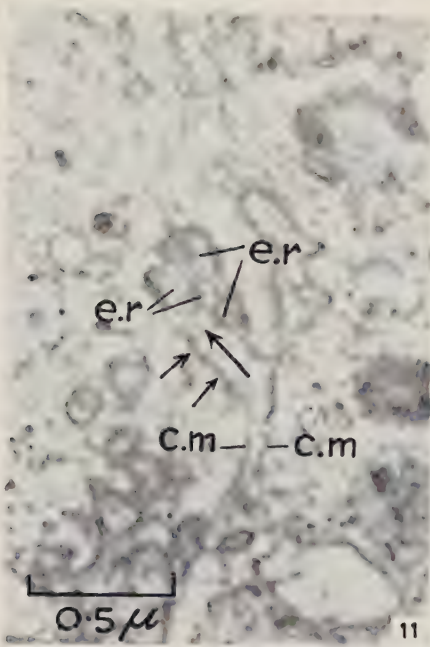
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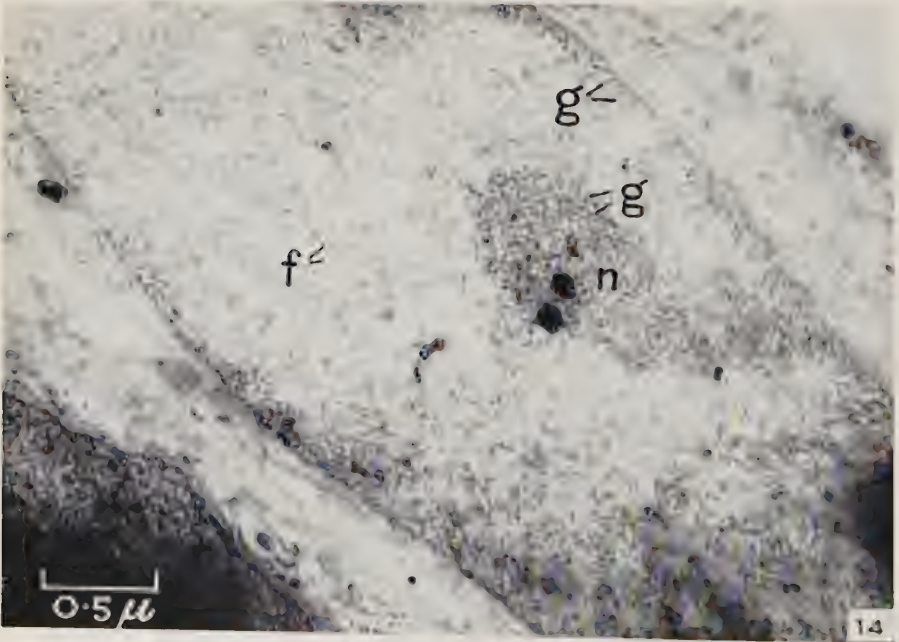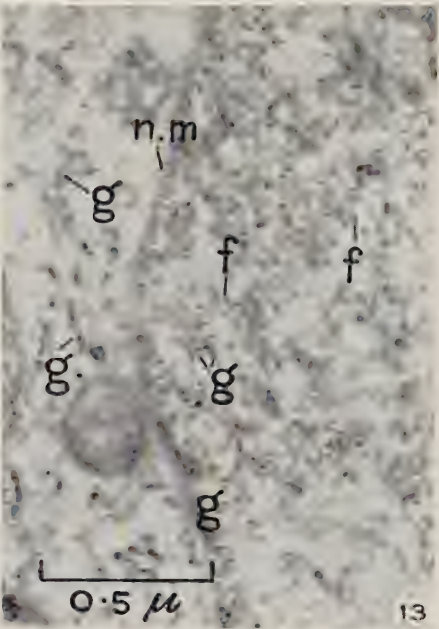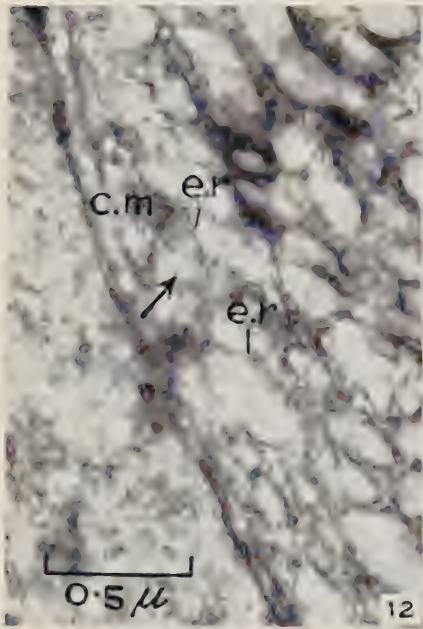
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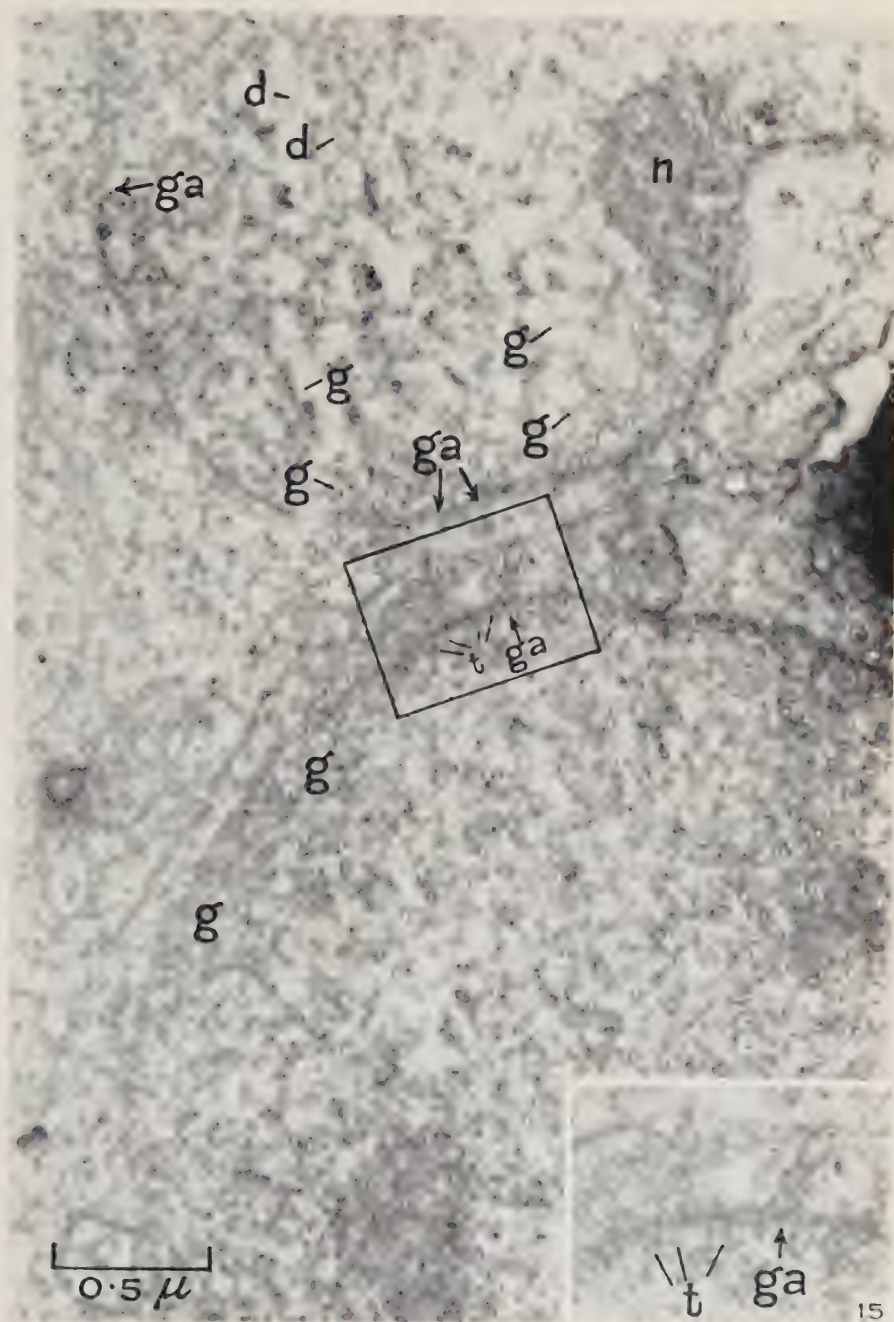
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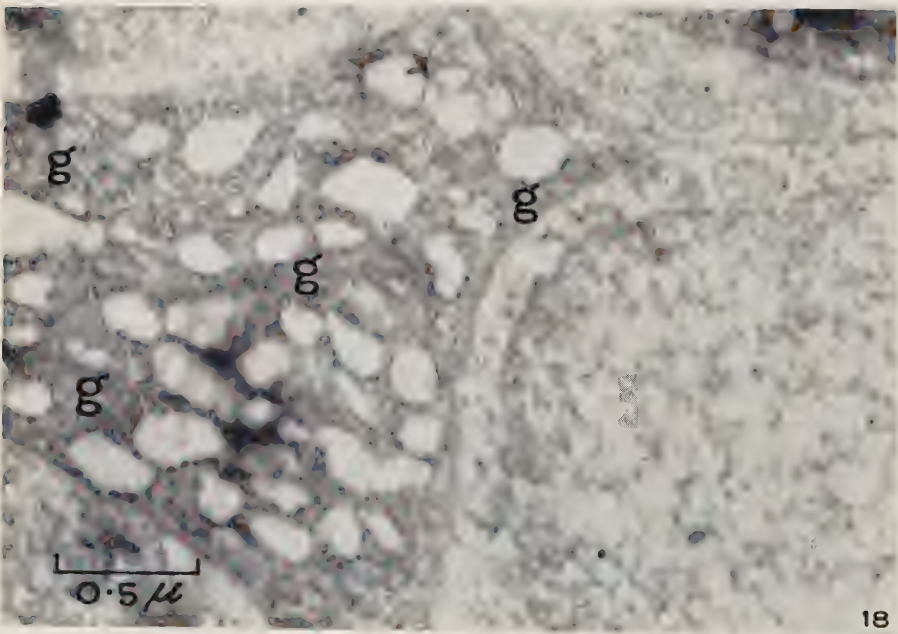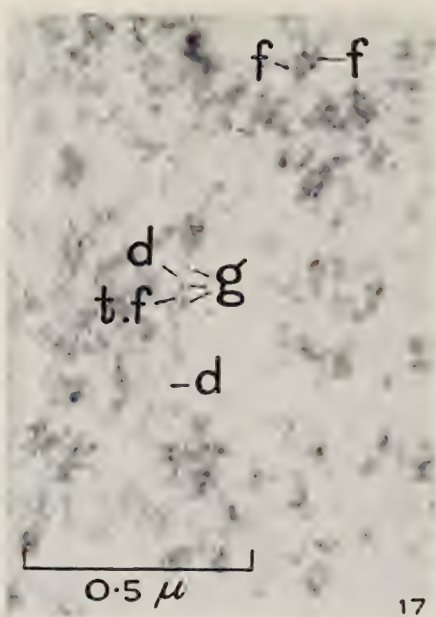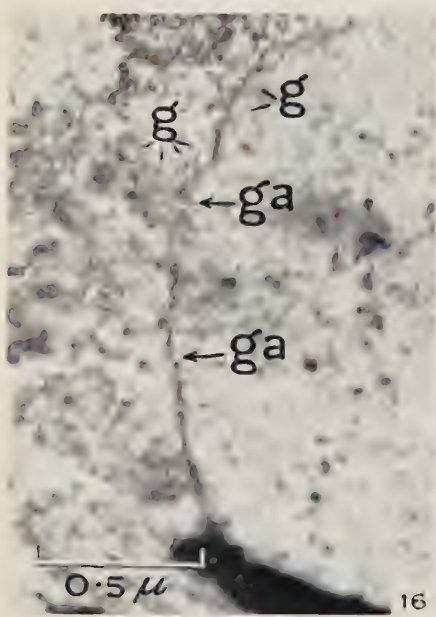
# STUDIES ON MARSUPIAL NUTRITION

## II. THE RATE OF PASSAGE OF FOOD RESIDUES AND DIGESTIBILITY OF CRUDE FIBRE AND PROTEIN BY THE QUOKKA, SETONIX BRACHYURUS (QUOY & GAIMARD)

### By J. H. CALABY*

*Summary*

The rate of passage of food residues through the digestive tract of a small grazing marsupial, *Setonix brachyurus* (Quoy & Gaimard) was determined. The animals were fed various mixtures of lucerne chaff, concentrates, and oaten chaff. The marked meal generally first appeared in the faeces 8–12 hr after feeding; 90 per cent. was excreted in about $1\frac{1}{2}$–$2\frac{1}{2}$ days and the meal was usually totally eliminated in 3–6 days. The rate of passage was slower with low food intake than with high food intake, and also with relatively coarser food. *S. brachyurus* excretes food residues in a pattern similar to that of domestic ruminants but at a faster rate, particularly in the terminal stages.

Digestibility of dry matter was 51–68 per cent., of crude fibre 25–48 per cent., and of crude protein 64–79 per cent. of the intake of the particular rations fed. When compared with domestic herbivores, fibre was digested considerably less efficiently by *S. brachyurus* than by ruminants but more efficiently than by the rabbit. The ability to digest crude protein was within the same range as for other herbivores for which figures are available. It is concluded from this work that *S. brachyurus* is intermediate in its digestive efficiency between the ruminants and non-ruminant herbivores.

## I. INTRODUCTION

Moir, Somers, and Waring (1956) have reported on the digestive physiology of the quokka, *Setonix brachyurus* (Quoy & Gaimard), a small grazing macropodid. The various features described—large sacculated stomach with an oesophageal groove, fermentation of food by bacteria with the production of organic acids, absorption of these acids, and low blood sugar levels—led these authors to conclude that the digestion pattern was ruminant-like.

Two associated characteristics of ruminant digestion are the long time food is retained in the tract and the efficient digestion of fibre. In the cow, sheep, and goat, for example, food residues are held in the rumen for several days, and it takes 2–4 days or more for 80 per cent. of the material to be excreted (Balch 1950; Blaxter, Graham, and Wainman 1956; Castle 1956a). In non-ruminant herbivores such as the horse, on the other hand, most if not all of the food residue is excreted in 24–48 hr (Alexander 1946). The digestibility of fibre is related to the time it spends in the digestive tract. Watson and Godden (1935) showed, for example, that the digestibility of crude fibre of artificially dried pasture was only 26·0 per cent. in the rabbit compared with 74·5 per cent. for the sheep.

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It was therefore of interest to investigate these related features of herbivore digestion in the quokka. A series of fibre-digestibility and rate-of-passage experiments using various diets is described in this paper. Crude protein digestibility and the nitrogen balance were also determined at the same time as fibre digestibility.

## II. EXPERIMENTAL

### (a) Animals

All experiments were done with wild male quokkas from Rottnest I. The animals were kept separately or in pairs, in yards equipped with small shelters, for a few weeks after capture to accustom them to humans and handling. The age was unknown but dental examination showed they were prime adults. It was presumed that all had a "normal" small infestation of the nematode *Austrostrongylus thylogale* Johnston & Mawson, which is found in quokkas taken on the island.

### (b) Cages and Collection of Excretion Products

The animals were kept individually in $1\frac{1}{4}$-in. mesh cages, 2 ft 6 in. long and 1 ft 3 in. square in cross section, placed on a rack about 3 ft above the floor. The quokka presented some difficulties for this type of investigation because of its habits of raking through the food and of carrying the food about in its fore paws. A special food container was devised to overcome this as much as possible. This consisted essentially of a small tin inside a large one with high sides and back, and with the front edges of both tins turned inwards. This arrangement prevented food spillage but there seemed no way of preventing food being carried away. This behaviour was subject to great individual variation and unsatisfactory animals were not used. A small piece of board on which the animals liked to rest was placed in each cage.

For the collection of faeces and urine, a long tray which sloped steeply from back to front was placed under each cage. The tray had a spout at the lower end and a piece of plastic tubing connected to it ran into a bottle. Covering the tray was a screen of fly-wire mounted on a wooden frame. The urine passed through the wire and the faecal pellets rolled down to the bottom end of the screen and were caught against a baffle board. The small amount of food dropped on to the screens was dried and weighed for computing food intake but was not used in samples for analysis.

Throughout the experiment the animals were weighed every two or three days, at the same time each day.

### (c) Diets

In the holding yards the animals were provided with sheep nuts* (a proprietary pelleted concentrate containing about 20 per cent. crude protein and $7\frac{1}{2}$ per cent. crude fibre) and water *ad lib.* From casual observations on caged animals it seems that a diet of sheep nuts alone may be detrimental to quokkas and some showed symptoms suggestive of ammonia poisoning. However, sufficient fibre was obtained from dried leaves, twigs, and bark of *Eucalyptus*, *Banksia*, etc. which littered the yards. On this abundant high protein diet the animals became very fat.

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*"Ewe and Lamb Sheep Nuts", manufactured by Hemphill Gray Oil Mills, Perth.

The quokkas could not be induced to eat any of the usual dried foodstuffs, such as grass or hay. In preliminary trials in cages, several samples of long or chaffed grass hay and oaten hay were offered but the animals ate very little and in fact would starve to death rather than eat the material. In one rate-of-passage experiment (RP1), a sample of oaten chaff in conjunction with sheep nuts and a small proportion of lucerne chaff, was eaten in sufficient amounts to maintain the weights of five out of six animals. Unfortunately no more of this material could be obtained for a digestibility trial. This particular sample was singularly free from insect and mould attack; all other samples of oaten chaff were slightly contaminated with one or the other.

Because of these difficulties the diets used were based on lucerne chaff which was readily accepted. The lucerne chaff was of good quality and contained 3·16 per cent. nitrogen and 27·6 per cent. crude fibre, determined on a dry weight basis. In experiment RP1 the animals were given 39 g of sheep nuts daily, all of which were eaten, and a mixture of seven parts of oaten chaff to one part of lucerne chaff *ad lib.* The food offered in experiments RP2 and D2 (D = digestion) was lucerne chaff alone. In experiments RP3 and D3 the food given was lucerne chaff intimately mixed with ground sheep nuts in the proportion of 3 : 1. The mixture contained 3·21 per cent. nitrogen and 25·1 per cent. crude fibre. The offering in experiments RP4 and D4 was equal parts of oaten chaff, lucerne chaff, and ground sheep nuts intimately mixed. Assay of the mixture gave 2·51 per cent. nitrogen and 20·8 per cent. crude fibre. These mixtures were chosen to vary the proportions of crude fibre and protein in the diet.

It was observed that quokkas often ejected a bolus of partly digested food which was re-eaten immediately. In the cages this fell through the wire and could not be recovered by the animal. There are no unequivocal observations of rumination by macropodids, but it is possible that the bolus is digesta which has been regurgitated in a manner analogous to the process in rumination. Quokkas were very variable in this behaviour. One individual produced a bolus every other day while others produced a bolus once in 10 days. It is possible that the regurgitation takes place more frequently than observed and that usually the bolus is not dropped from the mouth. During rate-of-passage experiments the bolus was discarded; however, it was fed back during digestibility trials and was almost always eaten immediately, even though the animal was not feeding at the time.

### (d) *Rate-of-passage Experiments*

For 3 weeks prior to the commencement of an experiment the animals were fed on the diet to be tested.

The method employed for measuring the rate of passage was an adaptation of that of Balch (1950) in which a small single meal stained with a dye is given to the animal and undigested dyed particles are identified visually in the faeces and counted. In all experiments quokkas were given 3–4 g of a moistened mixture of dyed chaff and a small amount of ground sheep nut. This was eaten in 10–20 min, the midpoint being taken as the starting time of the experiment. Brilliant green was the most satisfactory of a number of dyes tested.

Faeces were collected 8 hr later, every 4 hr for the next 2 days, and then at progressively longer intervals until no further coloured particles were being excreted. The total faeces for any collecting period were weighed, broken up and well mixed, and four 2·5-g samples were weighed out for counting. The preparation of the samples and counting technique were according to Balch. The total number of coloured particles excreted in each collection period was calculated and each period total was expressed as a percentage of the grand total. The cumulative percentages were plotted against time to give excretion curves.

TABLE 1

RATES OF EXCRETION OF FOOD RESIDUES BY QUOKKAS FED DIFFERENT DIETS

| Expt. No. and Diet | Animal No. | Body Weight (g) | Dry Matter Intake (g/day) | Time of: | | | |
|---|---|---|---|---|---|---|---|
| | | | | First Appearance (hr) | 5% Excretion (hr) | 90% Excretion (hr) | Last Appearance (hr) |
| RP1—39 g sheep | 1 | 3170 | 93 | 12 | 13 | 47 | 112 |
| nuts and 7 : 1 | 2 | 2650 | 62 | 16 | 18 | 66 | 140 |
| mixture of oaten | 3 | 3560 | 120 | 16 | 16 | 46 | 120 |
| and lucerne chaff | 4 | 3810 | 107 | 12 | 12 | 47 | 104 |
| *ad lib* | 5 | 3380 | 98 | 20 | 20 | 57 | 120 |
| | 6 | 3120 | 113 | 12 | 14 | 48 | 104 |
| RP2—lucerne chaff | 1 | 3460 | 116 | 8 | 12 | 34 | 104 |
| | 3 | 3480 | 138 | 12 | 12 | 36 | 68 |
| | 4 | 3900 | 112 | 8 | 14 | 38 | 88 |
| | 6 | 3340 | 119 | 12 | 17 | 42 | 96 |
| RP3—3 : 1 mixture | 7 | 3740 | 120 | 8 | 9 | 41 | 88 |
| of lucerne chaff and | 8 | 3230 | 112 | 12 | <12 | 43 | 88 |
| sheep nuts | 9 | 3850 | 124 | 8 | <8 | 39 | 88 |
| | 10 | 3780 | 108 | 12 | 13 | 43 | 72 |
| RP4—equal parts of | 1 | 3330 | 60 | 8 | 13 | 58 | 216* |
| oaten and lucerne | 4 | 3790 | 74 | 8 | 13 | 62 | 144 |
| chaff and sheep nuts | 8 | 3670 | 44 | 8 | 11 | 53 | 136 |
| | 10 | 3720 | 46 | 16 | 19 | 67 | 144 |

*In this animal the last appearance of coloured particles appeared to be 144 hr. However, a single coarse coloured particle which had apparently become caught in the tract was observed at 216 hr.

### (e) *Digestibility Trials*

At the end of the rate-of-passage experiment, a digestibility trial was started immediately, using the same animals and diet. The trial lasted 10 days. The excretion products were collected daily at feeding time (4 p.m.). The faeces were dried in an oven at 105°C and weighed when oven dry. The whole output for 10 days was broken up and mixed thoroughly and a sample taken for analysis. The 24-hr

urine sample was made up to 200 ml or 1 l. with water and sulphuric acid so that the final solution was 1N with respect to acid. 20-ml aliquots were taken daily, bulked, and stored at 0°C under toluene for analysis.

The unconsumed food was also collected daily and bulked, and at the conclusion of the experiment the total unconsumed food for each animal was weighed and a sample taken for analysis. This was necessary because some at least of the quokkas were able to effect a partial separation of the high protein components such as lucerne leaves and ground sheep nuts from the rest of the food.

The samples of food, unconsumed food, and faeces for analysis were ground in a Wiley mill and stored in air-tight jars.
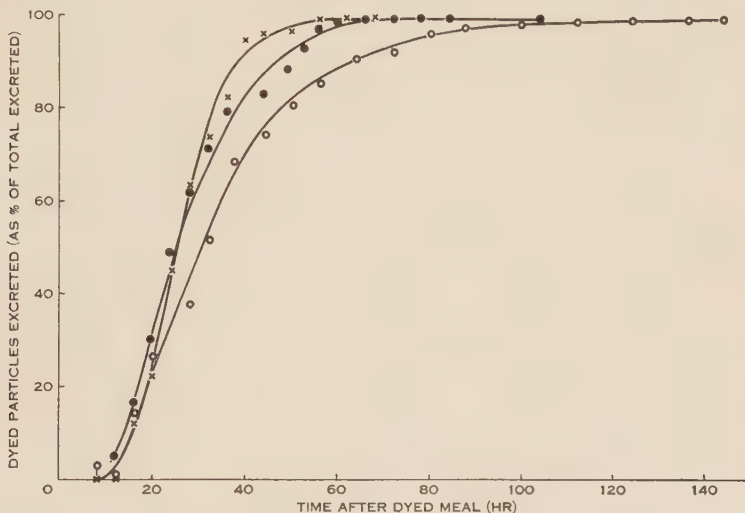


Fig. 1.—Rate-of-passage curves for one quokka (No. 4) fed different diets. ● Experiment RP1: daily intake of 39 g sheep nuts, plus 68 g of 7 : 1 oaten and lucerne chaff mixture. × Experiment RP2: daily intake of 112 g of lucerne chaff. ○ Experiment RP4: daily intake of 74 g of mixture of equal parts of oaten and lucerne chaff and sheep nuts.

### (f) Chemical Analyses

Nitrogen, crude fibre, and moisture analyses were done by standard methods recommended by the Association of Official Agricultural Chemists (1950). Nitrogen was determined in 1-g samples of food, unconsumed food, and faeces. 2-g samples were used for crude fibre and moisture analyses. For urinary nitrogen, 2-ml aliquots were taken when the daily sample had been made up to 200 ml and 10-ml aliquots when the daily sample had been made up to 1 l.

### III. Results

### (a) Rate-of-passage Experiments

Relevant data on the rate of passage of food residues are given in Table 1. The body weights recorded are the average weights of the animals during the course of the rate-of-passage experiment.

TABLE 2

DIGESTIBILITY COEFFICIENTS AND ASSOCIATED DATA FOR QUOKKAS FED DIFFERENT DIETS

| | Experiment D2—Lucerne Chaff | | | | Experiment D3—3:1 Mixture of Lucerne Chaff and Sheep Nuts | | | | Experiment D4—Equal Parts of Oaten and Lucerne Chaff and Sheep Nuts | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Animal No. | | | | Animal No. | | | | Animal No. | | | |
| | 1 | 3 | 4 | 6 | 7 | 8 | 9 | 10 | 1 | 4 | 8 | 10 |
| Dry matter intake (g/day) | 116·3 | 138·4 | 112·3 | 119·3 | 119·8 | 111·6 | 123·7 | 107·6 | 59·6 | 74·4 | 44·2 | 45·6 |
| Dry faeces (g/day) | 46·8 | 57·1 | 46·7 | 48·2 | 40·5 | 39·6 | 45·6 | 34·1 | 25·7 | 31·2 | 21·6 | 17·4 |
| Nitrogen intake (g/day) | 3·90 | 4·56 | 3·73 | 3·93 | 4·14 | 3·84 | 4·05 | 3·65 | 1·69 | 2·09 | 1·29 | 1·58 |
| Nitrogen in faeces (g/day) | 0·93 | 1·13 | 0·81 | 0·98 | 0·98 | 0·90 | 1·03 | 0·78 | 0·57 | 0·66 | 0·47 | 0·45 |
| Nitrogen in urine (g/day) | 2·17 | 2·50 | 2·25 | 1·97 | 2·39 | 2·25 | 2·25 | 2·63 | 1·33 | 1·36 | 1·42 | 1·34 |
| Nitrogen balance (g/day) | +0·80 | +0·94 | +0·67 | +0·98 | +0·78 | +0·70 | +0·77 | +0·24 | −0·21 | +0·07 | −0·60 | −0·21 |
| Crude fibre intake (g/day) | 29·7 | 35·6 | 28·6 | 29·8 | 23·4 | 25·0 | 29·9 | 24·1 | 11·9 | 11·3 | 8·9 | 8·7 |
| Dry matter digestibility (%) | 59·8 | 58·7 | 58·3 | 59·6 | 66·2 | 64·5 | 63·1 | 68·2 | 56·9 | 58·0 | 51·1 | 61·8 |
| Crude fibre digestibility (%) | 32·3 | 29·9 | 25·1 | 31·8 | 39·2 | 40·6 | 41·0 | 46·6 | 46·5 | 30·6 | 33·0 | 48·1 |
| Crude protein digestibility (%) | 76·2 | 75·2 | 78·3 | 75·1 | 76·3 | 76·6 | 74·6 | 78·6 | 66·3 | 68·4 | 63·6 | 67·3 |
| Weights of animals (g) | $3490^{+30}_{-0}$ | $3510^{+0}_{-30}$ | $3830^{+90}_{-90}$ | $3320^{+90}_{-0}$ | $3680^{+90}_{-0}$ | $3230^{+90}_{-0}$ | $3850^{+0}_{-110}$ | $3740^{+60}_{-0}$ | $3280^{+30}_{-20}$ | $3740^{+90}_{-0}$ | $3660^{+0}_{-430}$ | $3640^{+0}_{-60}$ |

All curves of the excretion of marked meals by quokkas are similar in form, and are of the same general shape as those of domestic ruminants (Balch 1950; Blaxter, Graham, and Wainman 1956; Castle 1956a).

The rate-of-passage curves obtained for one animal (No. 4) used in three experiments, are shown in Figure 1. The shape of each of these curves is typical for all of the animals in the corresponding experiments in which this animal was used. The rate of passage of this animal was one of the fastest in experiment RP1 but was intermediate in experiments RP2 and RP4.

### (b) Digestibility

Table 2 gives digestibility coefficients (i.e. the amount of nutrient digested, found by subtracting the content in the faeces from the amount in the intake, expressed as a percentage of the intake of the particular nutrient) and associated information on digestibilities and nitrogen balance. The weights of the animals at the start of the experiment are given, together with maximum departure from this weight during the 10 days of the experiment.

Within each experiment the dry matter and crude protein digestibilities of the animals are very similar. However, there is considerable variability between animals in their ability to digest crude fibre.

The crude protein digestibilities are lower in experiment D4 than in the two earlier experiments. The nitrogen content of this diet was much lower than in any of the previous diets.

### IV. DISCUSSION

It is of interest to compare the rate of passage through the digestive tract and digestibility coefficients in the quokka with published values on domestic ruminants and non-ruminant herbivores. The results are not strictly comparable as the nature and composition of the foods given are not the same. Nevertheless, some interesting comparisons may be made.

From an examination of results published by Balch (1950) who determined the rate of passage through dairy cows fed grass hay with or without various additions, it is seen that cows excrete food residues at a much slower rate than quokkas. Blaxter, Graham, and Wainman (1956) performed a series of tests with sheep fed different amounts of long, medium, or ground grass hay. All sheep on medium length grass, irrespective of the amount fed, have a slower rate of passage than the quokkas in experiment RP4; sheep fed long grass have an even slower rate.

The rates of passage for the well-fed quokkas in the first three experiments are fairly close to, or slightly slower than, the sheep having the fastest passage (those fed finely ground food in greatest amount) up to about 24–30 hr. After this time the passage through the sheep becomes considerably slower. The quokkas in experiment RP4 have a slower passage rate than these sheep up to about 36 hr. After this the sheep are close to the quokkas although the time before the marked meal is completely excreted is greater in the sheep.

Two of the factors influencing the rate of passage of food residues in the sheep, namely the degree of fineness and degree of fill, appeared to operate in the quokka.

TABLE 3

DIGESTIBILITY COEFFICIENTS FROM THE LITERATURE

| Species | Authority | Food | Dry Weight Analysis (%) | | Digestibility Coefficients (%) | | |
|---|---|---|---|---|---|---|---|
| | | | Crude Protein | Crude Fibre | Dry Matter | Crude Protein | Crude Fibre |
| Quokka | This paper | Lucerne chaff | 20·6–20·9 | 25·0–25·7 | 58·3–59·8 | 75·1–78·3 | 25·1–32·3 |
| | | Lucerne chaff and sheep nuts; lucerne and oaten chaff and sheep nuts | 17·6–21·6 | 15·2–24·2 | 51·1–68·2 | 63·6–78·6 | 30·6–48·3 |
| Sheep | Watson and Godden (1935) | Artificially dried pasture herbage | 20·8–20·9 | 21·3–21·6 | 74·3 | 76·4–76·9 | 73·5–74·5 |
| Sheep | Watson and Horton (1936) | Grass (several samples) | | 22 | 72·2–77·7 | 75·8–81·0 | 78·3–84·0 |
| Sheep | Louw (1944) | Lucerne hay | 14·9–18·4 | | 57·3–67·8 | 75·8–81·7 | |
| Sheep | Swift et al. (1950) | Lucerne | 23·9 | 25·1 | 61·9 | 74·5 | 48·9 |
| Cattle (dairy shorthorn cows) | Balch (1950) | Grass hay, variously ground, with or without concentrates | | | 58·2–70·8 | 47·8–67·3 | 36·3–74·8 |
| Cattle (dairy shorthorn cows) | Balch et al. (1953) | Lucerne hay | 13·8 | 38·2 | 56·7–63·5 | 68·1–73·4 | 53·5–62·8 |
| Cattle (Holstein heifers) | Swanson and Herman (1952) | Lucerne, variously ground | 14·8 | 35·8 | 54·1–60·0 | 67·6–73·2 | 37·9–48·8 |
| Cattle (steers) | Crampton et al. (1940) | Chaffed pasture herbage | 27·1–29·5 | 15·0–17·6 | 62–70 | 72–77 | 65–77 |
| Cattle (Afrikaner steers) | Groenewald et al. (1950, 1952) | Lucerne hay | 17·1–18·3 | 29·2–30·7 | 54·8–63·9 | 71·3–77·3 | 32·0–49·3 |
| Deer (Odocoileus hemionus (Rafinesque)) | Bissell et al. (1955) | Lucerne hay | 14·3–15·9 | | | 67·9–75·1 | |
| Rabbit | Voris et al. (1940) | Lucerne chaff | 20·1–30·3 | 21·8–34·9 | 48·1–67·5 | 72·3–83·6 | 18·2–29·8 |
| Rabbit | Watson and Godden (1935) | Artificially dried pasture herbage | 20·8–20·9 | 21·3–21·6 | 45·6–50·9 | 60·8–64·3 | 21·3–28·7 |
| Rabbit | Watson and Horton (1936) | Grass (several samples) | | 22 | 62·8–63·4 | 76·2–79·0 | 46·9–51·0 |
| Rabbit | Crampton et al. (1940) | Chaffed pasture herbage | 27·1–29·5 | 15·0–17·6 | 45–53 | 65–70 | 24–31 |

The 90 per cent. elimination times were longer in experiment RP1 where the material fed was relatively coarser, and also in experiment RP4 where the intakes were considerably reduced below those in experiments RP2 and RP3.

Castle (1956a) has worked with goats using meadow hay and concentrates as food. Generally the rate of passage in goats was slower than in quokkas. Castle quotes earlier workers who obtained much slower rates of passage in goats and in later papers (1956b, 1956c) obtained somewhat slower rates herself with a few animals.

Little information is available on the rate of passage of food residues in non-ruminant herbivores. Alexander (1946) experimented with horses fed oats and bran using carbon granules as a marker. He found that the marker appeared in the faeces 22 hr after feeding and was completely eliminated by 48 hr (means of 17 separate observations on five different animals). The only experiment on the rabbit known to the author (cf. Elliott and Barclay-Smith 1904) is not comparable since the glass beads used do not fulfil the requirements of a satisfactory marker (Alexander 1946).

It is evident that undigested food residues are excreted by the quokka in a manner similar to that of ruminants but at a faster rate particularly in the later stages. The slow elimination in the later stages is a characteristic of ruminants.

In Table 3, digestibility coefficients of standard feeding stuffs, particularly lucerne, from experiments on the quokka, ruminants, and the rabbit, are listed from the literature. The relative digestion efficiency of the rabbit and ruminants is best illustrated by the work of C. J. Watson and Godden (1935), S. J. Watson and Horton (1936), and Crampton, Campbell, and Lange (1940) who compared sheep or cattle with rabbits fed identical diets. In these comparative studies the protein digestion coefficients for the ruminants are consistently higher than those for the rabbits. From the values in Table 3 it may be seen that all species (including the quokka) digest the crude protein of good quality diets efficiently.

The relative ability of ruminant and non-ruminant herbivores to digest crude fibre is reasonably clear-cut, and in this regard the quokka is considerably less efficient than ruminants, but more efficient than the rabbit. Morrison (1938) states that the horse is somewhat less efficient than ruminants at digesting feed, particularly fibre. Alexander (1952) using the *in vivo* cotton-thread technique of Balch and Johnson (1950) showed that the capacity of the organisms in the large intestine of the horse to ferment cellulose is not inferior to those of the rumen of the cow. The degree of digestion therefore depends upon the time for which the cellulosic material is exposed to the fermentation.

In ruminants, the time taken by the food to pass through the tract is largely spent in the rumen where the food undergoes active digestion. Because of differences in stomach anatomy and presumably movements, the pathway of food in the rumen is more complex than in the simpler stomach of the quokka. No determination was made of the time the food was in the quokka stomach but it is probably appreciable in terms of the total length of the alimentary tract, assuming that the digestive efficiency of the ruminant rumen is similar to that of the quokka stomach plus caecum. The fact that digestion of fibre by the quokka is intermediate between the two herbivore types suggests, too, that the length of stay of food in the quokka stomach

is longer than in the caecum, assuming that the rate of movement through the rest of the gut is of the same order in the two types.

In the quokka, not only is the rate of passage faster than in ruminants, but the digestibility of fibre is considerably lower. The quokka therefore occupies an intermediate position between the ruminants and non-ruminant herbivores with regard to the related characteristics—rate of passage and efficient digestion of fibre.

## V. Acknowledgments

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# THE EFFECTS OF INCUBATION TEMPERATURE AND COLD SHOCK ON THE METABOLISM OF RAM SPERMATOZOA

By A. W. Blackshaw*

*Summary*

The glycolysis of ram semen was measured at 37, 21, and 5°C. Measurements of $Q_{10}$ over this range gave values between about 2 and 3 for changes in lactic acid and total reducing substances. Cold shock greatly reduced glycolysis and dehydrogenase activity which were only partially maintained by egg yolk and lecithin.

## I. INTRODUCTION

The motility of spermatozoa may be reduced by decreasing the environmental temperature and this has been used as a means of storing semen for artificial insemination. The metabolic activity of semen may be expected to show a similar decline to motility and a few reports have shown that this does occur (Gladcinova 1936; Moore and Mayer 1941; Mann 1946; Blackshaw, Salisbury, and VanDemark 1957).

Linked with the effects of low temperature on metabolic functions is the phenomenon of cold or temperature shock produced by a rapid fall in temperature, and characterized by irreversible loss of motility. It has been shown by Walton (1947), Kampschmidt, Mayer, and Herman (1953), and Blackshaw (1954a) that various lipid constituents of egg yolk as well as the yolk itself are of value in preventing cold shock.

Metabolic studies of the consequences of cold shock are few, but indicate that metabolism is severely affected and the normal permeability of the cells is destroyed (Walton 1942; Mayer 1955; Mann and Lutwak-Mann 1955; Blackshaw and Salisbury 1957). This paper describes experiments in which the glycolysis of ram spermatozoa was measured at 5, 21, and 37°C. The effects of cold shock on the metabolic rate and the protective action of egg yolk and lecithin were also studied.

## II. MATERIALS AND METHODS

Ram semen was collected by the electrical stimulation of ejaculation (Blackshaw 1954b) and was used for the metabolic studies within 3 hr.

The diluents used in all tests were modifications of that described by White (1953). At pH 7·0 the diluent contained 0·048M $Na_2HPO_4.12H_2O$, 0·032M $NaH_2PO_4.2H_2O$, and 0·040M NaCl and 200 mg per cent. fructose. At pH 7·5 the

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amounts of $Na_2HPO_4.12H_2O$ and $NaH_2PO_4.2H_2O$ were 0·058M and 0·018M respectively and at pH 6·5, 0·022M and 0·066M. In some experiments egg yolk was added to the diluent to give a concentration of 20 or 50 per cent. (v/v).

TABLE 1

EFFECT OF INCUBATION TEMPERATURES AND THE PRESENCE OF EGG YOLK ON THE METABOLISM AND MOTILITY OF RAM SPERMATOZOA

Each result is the mean of 9 ejaculates

| Temperature (°C) | Egg Yolk (%) | Final Motility | pH | (A) Decrease in Total Reducing Substances ($\mu g/10^8$ cells/hr) | (B) Increase in Lactic Acid ($\mu g/10^8$ cells/hr) | Ratio B/A |
|---|---|---|---|---|---|---|
| 37 | 0 | 2·6 | 6·60 | 121 | 97 | 0·80 |
|    | 20 | 1·7 | 6·40 | 140 | 100 | 0·71 |
| 21 | 0 | 2·3 | 6·75 | 25 | 21 | 0·84 |
|    | 20 | 2·1 | 6·65 | 52 | 30 | 0·58 |
| 5  | 0 | 2·4 | 6·85 | −8 | 4 | — |
|    | 20 | 2·6 | 6·70 | 1 | 14 | 14·00 |

Analyses of Variance

| Source of Variation | Degrees of Freedom | Total Reducing Substances | | Lactic Acid | |
|---|---|---|---|---|---|
|  |  | Mean Square | Variance Ratio | Mean Square | Variance Ratio |
| Ejaculates (1) | 8 | 2,376 | 12·2** | 6,796 | 60·7** |
| Yolk (2) | 1 | 4,630 | 23·8** | 690 | 6·2* |
| Temperature (3) | 2 | 84,444 | 436·2** | 40,631 | 363·0** |
| Interactions |  |  |  |  |  |
| 1 × 2 | 8 | 1,540 | 7·9* | 670 | 6·0** |
| 1 × 3 | 16 | 1,497 | 7·7* | 1,957 | 17·5** |
| 2 × 3 | 2 | 2,855 | 14·6** | 58 | 0·5 |
| Residual | 16 | 194·5 |  | 111·9 |  |

*$P < 0.05$. **$P < 0.01$.

A partially purified preparation of lecithin was made by extracting the acetone-insoluble fraction of egg yolk in a blendor with 95 per cent alcohol. This was vacuum-dried, dissolved in ether, and precipitated by acetone in which it was stored. A 1 per cent. (w/v) suspension of the extract dried under vacuum was used in the tests.

For the experiments on temperature effects the neat semen was slowly cooled to 5°C and diluted 1 to 4 with the test diluent. Samples were taken for the deter-

mination of initial lactic acid and reducing sugar levels. The cold, diluted semen was then rewarmed to the required incubation temperature. The period of incubation was 4 hr at 37°C and 8 hr at 21 and 5°C.

To produce cold shock, 1·5 ml of semen, diluted at room temperature, was chilled by plunging into an ice-bath for 10 min. Samples were taken from the chilled and control tubes for chemical analysis immediately before incubation at 37°C and at the end of 3 hr.

Lactic acid was estimated by the method of Barker and Summerson (1941) and total reducing substances by a method using the chromogenic mixture of Nelson (1944) and the reducing agent of Somogyi (1952).

TABLE 2

$Q_{10}$ FOR LACTIC ACID ACCUMULATION AND THE CHANGE IN TOTAL REDUCING SUBSTANCES

Results from means of 9 ejaculates

| Temperature Range (°C) | Egg Yolk (%) | Lactic Acid Accumulation | Change in Total Reducing Substances |
|---|---|---|---|
| 37–21 | 0 | 2·6 | 2·7 |
|  | 20 | 2·1 | 1·9 |
| 37–5 | 0 | 2·7 | — |
|  | 20 | 1·9 | 4·8 |

The effect of cold shock on the dehydrogenase activity of semen was estimated by the reduction of 2,3,5-triphenyltetrazolium chloride (Kun and Abood 1949). The diluted semen (1 : 4) in 1·5-ml amounts was mixed with sufficient dye to give a concentration of 0·25 mg/ml. A layer of liquid paraffin (2 ml) was added and the tubes incubated for 2 hr at 37°C in the dark. One-ml aliquots of semen were removed, mixed with 7 ml of acetone, and centrifuged to obtain a clear supernatant. The optical density was measured at 495 $\mu$ in a Coleman model 14 spectrophotometer.

The motility of spermatozoal suspensions was scored by the method of Emmens (1947) in which maximum motility is scored as 4 and complete immotility as 0.

The $Q_{10}$ values were calculated from the following equation:

$$\log Q_{10} = \frac{10 \ (\log k_1 - \log k_2)}{(t_1 - t_2)},$$

where $k_1$ and $k_2$ are the velocity constants at the temperatures $t_1$ and $t_2$.

III. RESULTS

The effects on metabolism of incubation temperatures of 37, 21, and 5°C are given in summary form (Table 1). Analysis of variance of both the lactic acid and reducing sugar changes (Table 1) showed that egg yolk increased the metabolic rate

but the effect was small. The mean values from Table 1 were used to calculate $Q_{10}$ for the temperature ranges of 37–21°C and 37–5°C (Table 2).

Further tests were made to observe the effects of cold shock on the motility and metabolism of ram spermatozoa. The influence of 20 per cent. (v/v) egg yolk on glycolysis following shock was measured at 37°C (Table 3). In this series the yolk

TABLE 3

EFFECT OF COLD SHOCK ON THE METABOLISM AND MOTILITY OF RAM SPERMATOZOA

Results are means of 9 ejaculates

| Treatment | Egg Yolk (%) | Final Motility | (A) Decrease in Total Reducing Substances ($\mu g/10^8$ cells/hr) | (B) Increase in Lactic Acid ($\mu g/10^8$ cells/hr) | Ratio B/A |
|---|---|---|---|---|---|
| Control | 0 | 3·0 | 137 | 115 | 0·87 |
|  | 20 | 2·7 | 129 | 96 | 0·74 |
| Shock | 0 | 0·0 | —9 | 9 | — |
|  | 20 | 1·5 | 34 | 47 | 1·40 |

Analyses of Variance

| Source of Variation | Degrees of Freedom | Total Reducing Substances | | Lactic Acid | |
|---|---|---|---|---|---|
|  |  | Mean Square | Variance Ratio | Mean Square | Variance Ratio |
| Ejaculates (1) | 8 | 3,686 | 2·5 | 3,504 | 3·3* |
| Shock (2) | 1 | 132,860 | 89·3** | 53,592 | 50·2** |
| Yolk (3) | 1 | 2,584 | 1·7 | 702 | 0·7 |
| Interactions |  |  |  |  |  |
| 1 × 2 | 8 | 2,574 | 1·7 | 1,685 | 1·6 |
| 1 × 3 | 8 | 383 | 0·3 | 923 | 0·9 |
| 2 × 3 | 1 | 5,256 | 3·5 | 7,253 | 6·8* |
| Residual | 8 | 1,488 |  | 1,067 | — |

*$P < 0·05$.    **$P < 0·01$.

did not affect the metabolism of the control semen and only the lactic acid production of shocked semen was significantly improved (Table 3) by egg yolk. Similar results were obtained when 1 per cent. (w/v) lecithin was used in place of yolk; the pH of the medium did not affect the results with shocked semen but at pH 6·5 lactic acid accumulation in the controls was reduced (Table 4).

The effect of different temperatures on the development of cold shock was studied using 50 per cent. egg yolk and 1 per cent. lecithin as protective agents.

The diluted semen was cooled from $30 \cdot 0$ to $22 \cdot 5$, $15 \cdot 0$, $7 \cdot 5$, and $0°C$ and held at these temperatures for 10 min and then incubated at $37°C$ for 3 hr. The mean lactic acid changes for seven ejaculates and summary analyses of variance for each diluent are given in Table 5.

<div align="center">

TABLE 4

EFFECT OF LECITHIN ON THE ACCUMULATION OF LACTIC ACID BY RAM SPERMATOZOA
AFTER COLD SHOCK

</div>

| Lecithin (% w/v) | Control pH | Lactic Acid Accumulation ($\mu$g/$10^8$ cells/hr) | Shock pH | Lactic Acid Accumulation ($\mu$g/$10^8$ cells/hr) |
|---|---|---|---|---|
| 0 | $6 \cdot 5$ | 66 | $6 \cdot 5$ | 11 |
|   | $7 \cdot 5$ | 99 | $7 \cdot 5$ | 15 |
| 1 | $6 \cdot 5$ | 78 | $6 \cdot 5$ | 39 |
|   | $7 \cdot 5$ | 95 | $7 \cdot 5$ | 34 |

<div align="center">Analyses of Variance</div>

| Source of Variation | Degrees of Freedom | Control | | Shock | |
|---|---|---|---|---|---|
|  |  | Mean Square | Variance Ratio | Mean Square | Variance Ratio |
| Ejaculates (1) | 5 | 4641 | $18 \cdot 8$** | 564 | $0 \cdot 9$ |
| Lecithin (2) | 1 | 85 | $0 \cdot 3$ | 3197 | $5 \cdot 0$* |
| pH (3) | 1 | 3798 | $15 \cdot 4$** | 0 | $0 \cdot 0$ |
| Interactions |  |  |  |  |  |
| $1 \times 2$ | 5 | 161 | $0 \cdot 6$ | 242 | $0 \cdot 4$ |
| $1 \times 3$ | 5 | 1040 | $4 \cdot 2$ | 614 | $0 \cdot 9$ |
| $2 \times 3$ | 1 | 369 | $1 \cdot 5$ | 126 | $0 \cdot 2$ |
| Residual | 5 | 246 |  | 640 |  |

*$P < 0 \cdot 05$.  **$P < 0 \cdot 01$.

The damage produced by cold shock on the dehydrogenase systems of ram spermatozoa was demonstrated by the reduction of 2,3,5-triphenyltetrazolium chloride. Preliminary tests with normal ram semen showed that the reduction was slow aerobically but under a layer of liquid paraffin reduction proceeded rapidly. This technique was used in the experimental procedure.

After cold shock the semen was incubated at $37°C$ for 2 hr with the dye and the colour extracted with acetone. The mean values for the optical density readings are given in Table 6 for both egg yolk and lecithin. The analyses of variance (Table 6) indicate that both substances partially prevented the decline in dehydrogenase activity produced by cold shock.

## IV. Discussion

The metabolic activity of ram sperm is greatly decreased by a reduction in incubation temperature and the $Q_{10}$ obtained show that the effect is similar to that found in other tissues in which a $Q_{10}$ of between 2 and 3 is common. It is clear that ram spermatozoa are very susceptible to rapid temperature changes and that egg

TABLE 5

EFFECT OF DILUENT AND THE DEGREE OF COLD SHOCK ON THE ACCUMULATION OF
LACTIC ACID BY RAM SPERMATOZOA

Results are means of 7 ejaculates

| Shock Temperature (°C) | Lactic Acid Accumulated ($\mu$g/$10^8$ cells/hr) | | |
| --- | --- | --- | --- |
| | Control | 50% Egg Yolk | 1% Lecithin |
| 30·0 | 98 | 114 | 106 |
| 22·5 | 53 | 73 | 116 |
| 15·0 | 67 | 72 | 106 |
| 7·5 | 26 | 69 | 75 |
| 0·0 | 8 | 39 | 5 |

Analyses of Variance

| Source of Variation | Degrees of Freedom | Variance Ratios | | |
| --- | --- | --- | --- | --- |
| | | Control | Egg Yolk | Lecithin |
| Ejaculates | 6 | 13·7** | 4·7* | 42·3** |
| Temperature | (4) | | | |
| Linear | 1 | 25·8** | 9·8** | 17·0** |
| Quadratic | 1 | 0·0 | 0·1 | 6·8* |
| Cubic | 1 | 0·8 | 1·8 | 0·1 |
| Quartic | 1 | 3·2 | 0·0 | 0·0 |
| Residual | 24 | 1180† | 1671† | 2430† |

*$P<0·05.$  **$P<0·01.$
†Residual mean squares.

yolk and lecithin do not give sufficient protection against severe cold shock to enable the spermatozoa to maintain their full metabolic activities during a subsequent period of incubation at 37°C.

Over a temperature shock range from 30 to 0°C the decline in metabolic activity was linear for both the control semen and that diluted with the yolk medium but at 7·5°C the semen in the lecithin diluent showed an abrupt decline in lactic acid accumulation not seen in the semen in egg yolk.

These observations are in marked constrast to those described for bull semen by Blackshaw and Salisbury (1957). In this species egg yolk and lecithin were both highly effective in preventing the occurrence of cold shock and also in maintaining the metabolism of the semen during subsequent incubation at 37°C. In addition, yolk stimulated the glycolysis of unshocked semen.

TABLE 6

EFFECT OF EGG YOLK AND LECITHIN ON THE OCCURRENCE OF COLD SHOCK AS SHOWN BY THE REDUCTION OF 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE

Results given as mean optical densities

|  | Egg Yolk (% v/v) (mean of 6 ejaculates) | Optical Density | Lecithin (% w/v) (mean of 9 ejaculates) | Optical Density |
|---|---|---|---|---|
| Control | 0 | 0·396 | 0 | 0·256 |
|  | 20 | 0·262 | 1 | 0·250 |
| Shock | 0 | 0·202 | 0 | 0·106 |
|  | 20 | 0·231 | 1 | 0·188 |

Analyses of Variance

| Source of Variation | Degrees of Freedom | Variance Ratio Egg Yolk | Degrees of Freedom | Variance Ratio Lecithin |
|---|---|---|---|---|
| Ejaculates | 5 | 9·00** | 8 | 15·35** |
| Shock | 1 | 20·83** | 1 | 34·8** |
| Agent | 1 | 4·54 | 1 | 4·56* |
| Shock × agent | 1 | 10·98** | 1 | 6·20* |
| Error | 15 | 0·0036† | 24 | 0·0028† |

*$P < 0·05$.   **$P < 0·01$.

†Error mean squares.

The usual temperature for the storage of semen for artificial insemination has been 5°C, but it has been indicated (Emmens and Blackshaw 1956) that the fertility of stored ram semen is low. Recently it has been shown that at 20°C, in the presence of $CO_2$, the fertile life of bull semen could be extended to 1 week (VanDemark and Sharma 1957). In view of the high sensitivity of ram spermatozoa to cold shock and the relative inefficiency of protective agents, the use of this method of storage for ram semen at 20°C may be preferable.

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# THE EFFECT OF ALKALI METAL, MAGNESIUM, AND CALCIUM IONS ON THE MOTILITY OF FOWL SPERMATOZOA

By R. G. Wales* and I. G. White*

*Summary*

Potassium (5–45 mM), magnesium (1·5–13·5 mM), and calcium (0·3–2·7mM) chlorides each increase the viability of fowl spermatozoa *in vitro* when added to a diluent composed of 0·02M sodium phosphate buffer, 0·5 per cent. sodium chloride, and 1·5 per cent. glucose.

Additive effects were given by potassium in combination with magnesium or calcium but motility in the diluent containing magnesium and calcium was less than would be expected from the sum of their separate effects.

Calcium ions in concentrations greater than 0·3 mM caused agglutination of the spermatozoa.

Washing twice or more was harmful to fowl spermatozoa and the effect of potassium was relatively greater on the washed than the unwashed cells.

Rubidium ions were as effective as potassium ions in stimulating motility, caesium ions were ineffective, and lithium and ammonium ions were toxic.

Potassium levels in excess of 180 mM depressed the motility of fowl spermatozoa and diluents composed entirely of potassium salts are therefore undesirable.

## I. Introduction

Potassium, magnesium, and calcium occur in appreciable quantities in semen (see Mann 1954) and attention has recently been drawn to the effect of these ions on spermatozoa (White 1956).

The importance of potassium for the normal functioning of ram and bull spermatozoa can be readily demonstrated by comparing their viability after repeated washing in potassium-containing and potassium-free media (Lardy and Phillips 1943; White 1953*a*, 1953*b*; Blackshaw 1953*a*, 1953*b*). Viability remains high in the solution containing potassium but the spermatozoa become immotile after 3 hr at 37°C in its absence. Studies of the capacity of the rarer alkali metals to replace potassium in washed ram and bull spermatozoa show an interesting gradation in biological properties which can be readily correlated with their sequence in the periodic tables (White 1953*c*).

According to Lardy and Phillips (1943) magnesium improves the motility and glycolysis of washed bull spermatozoa; calcium on the other hand has been found to decrease the viability of ram and bull spermatozoa (Lardy and Phillips 1943; Blackshaw 1953*a*).

There appears to be little or no information on the ionic requirements of fowl spermatozoa and it is the purpose of this paper to report studies on the effect of the alkali metals, magnesium, and calcium ions on the spermatozoa of this species.

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## II. Materials and Method

Fowl semen was obtained by abdominal massage (Burrows and Quinn 1939). Only apparently normal specimens of good initial motility were employed.

Unwashed spermatozoal suspensions were prepared by diluting 0·5 ml of semen with 4·5 ml of diluent in a graduated centrifuge tube, and washed spermatozoa were prepared by centrifuging this suspension at 1500 r.p.m. (about 300 $g$) for 10 min, the supernatant being drawn off and replaced by diluent after each centrifuging and the spermatozoa re-dispersed by sucking up and down in a wide-bore pasteur pipette fitted with a rubber teat. Centrifuged but unwashed spermatozoal suspensions were prepared by centrifuging the diluted (1 in 10) semen and re-dispersing the cells after each centrifuging without removing the supernatant.

Table 1

CONCENTRATIONS OF POTASSIUM, MAGNESIUM, AND CALCIUM IN DILUENTS

| Metal | Level 1 (mM) | Level 2 (mM) | Level 3 (mM) |
|---|---|---|---|
| Potassium | 5 | 15 | 45 |
| Magnesium | 1·5 | 4·5 | 13·5 |
| Calcium | 0·3 | 0·9 | 2·7 |

The diluted semen was pipetted into small tubes and kept at room temperature. For the determination of motility, a drop of spermatozoal suspension was placed on a glass slide and examined under the microscope. Motility was scored by the system of Emmens (1947). Full motility was rated as four and complete immotility as zero, but in presenting the results the actual scores have been multiplied by 4, since quarter-grades were frequently used. The sum of the motility scores multiplied by 4 for each ejaculate over the experimental period has been used as unit observation (see Emmens 1948) in the analyses of variance which are presented in summary form.

All diluents were isotonic and of pH 7·0. They were prepared from A.R. chemicals and stored in a deep-freeze cabinet at $-80°C$ between experiments. The control diluent had the following composition: 0·02M sodium phosphate buffer, 0·5 per cent. sodium chloride, 1·5 per cent. glucose. Potassium, magnesium, and calcium were added as the chloride salts at the concentrations shown in Table 1 and the sodium chloride content adjusted to keep the diluent isotonic.

## III. Results

### (a) Preliminary Experiments

The technique of washing mammalian spermatozoa has been widely used in studying their metabolism and ionic requirements. The damage caused to ram and bull spermatozoa by the process has been investigated by White (1953d).

As no studies of the effect of washing on avian spermatozoa have been reported, the effect of washing and of sedimentation by centrifuging on fowl spermatozoa was studied by comparing the motility of the unwashed cells with that of washed suspensions and with spermatozoa centrifuged without washing. The washing and centrifuging procedures were repeated in each case twice and four times. Two diluents used were (i) the control and (ii) the control plus level 1 (see Table 1)

TABLE 2

EFFECT OF WASHING AND CENTRIFUGING ON THE MOTILITY INDICES OF FOUR FOWL EJACULATES

| Treatment | Diluent A (control) | | | | | Diluent B (control + level 1 of potassium and magnesium) | | | | | Totals |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Total | 1 | 2 | 3 | 4 | Total | |
| Diluted | 45 | 58 | 62 | 55 | 220 | 58 | 61 | 63 | 63 | 245 | 465 |
| Washed twice | 25 | 40 | 25 | 10 | 100 | 45 | 60 | 53 | 29 | 187 | 287 |
| Centrifuged twice | 39 | 55 | 62 | 52 | 208 | 52 | 58 | 60 | 56 | 226 | 434 |
| Washed four times | 22 | 35 | 17 | 6 | 80 | 43 | 59 | 53 | 23 | 178 | 258 |
| Centrifuged four times | 32 | 48 | 61 | 45 | 186 | 45 | 60 | 62 | 54 | 221 | 407 |
| Totals | 163 | 236 | 227 | 168 | 794 | 243 | 298 | 291 | 225 | 1057 | 1851 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | Variance Ratio | |
|---|---|---|---|
| Between diluents | 1 | 86·5** | |
| Between ejaculates | 3 | 27·1** | |
| Between treatments | 4 | 53·0** | |
|   Washing v. centrifuging | 1 | | 136·8** |
|   Washing twice v. washing four times | 1 | | 2·7 |
|   Centrifuging twice v. centrifuging four times | 1 | | 2·3 |
|   Treatment v. controls | 1 | | 70·2** |
| Interactions | | | |
|   Diluent × treatment | 4 | 8·6** | |
|   Diluent × ejaculate | 3 | 0·5 | |
|   Treatment × ejaculate | 12 | 5·7** | |
| Residual | 12 | 20 | |

** $P < 0.01$.

of potassium and magnesium since both ions had proved beneficial to mammalian spermatozoa, particularly after washing. Motility was scored at 2, 4, 8, and 16 hr from the start of each test.

The results for four ejaculates and the analysis of variance are given in Table 2. Motility in the diluent containing potassium and magnesium was significantly better,

especially after washing. Partitioning of the treatment sum of squares showed that washing was more harmful than merely centrifuging. Clearly the damage done

TABLE 3

EFFECT OF POTASSIUM, MAGNESIUM, AND CALCIUM ON THE MOTILITY OF UNWASHED AND TWICE-WASHED FOWL SPERMATOZOA

Each value represents the mean motility index for six ejaculates

| | Unwashed | | | | Washed | | | | Grand Mean |
|---|---|---|---|---|---|---|---|---|---|
| Ions Added | Level 1 | Level 2 | Level 3 | Mean | Level 1 | Level 2 | Level 3 | Mean | |
| Nil | 42·0 | 44·3 | 44·2 | 43·5 | 23·5 | 24·2 | 21·8 | 23·2 | 33·4 |
| Potassium | 46·2 | 48·7 | 41·7 | 45·6 | 29·7 | 27·8 | 22·7 | 26·7 | 36·1 |
| Magnesium | 50·7 | 48·0 | 50·0 | 49·6 | 29·5 | 29·8 | 32·3 | 30·6 | 40·1 |
| Calcium | 47·5 | 45·5 | 49·0 | 47·3 | 25·5 | 27·0 | 27·0 | 26·5 | 36·9 |
| Potassium, magnesium | 50·8 | 51·3 | 47·8 | 50·0 | 32·9 | 37·7 | 34·0 | 34·8 | 42·4 |
| Potassium, calcium | 50·0 | 50·0 | 53·0 | 51·0 | 30·8 | 33·8 | 31·3 | 32·0 | 41·5 |
| Magnesium, calcium | 49·0 | 51·0 | 51·7 | 50·6 | 28·2 | 30·2 | 28·5 | 28·9 | 39·8 |
| Potassium, magnesium, calcium | 52·3 | 52·0 | 51·2 | 51·8 | 35·2 | 33·0 | 38·3 | 35·5 | 43·7 |

Analysis of Variance

| Source of Variation | D.F. | Variance Ratio | Source of Variation | D.F. | Variance Ratio |
|---|---|---|---|---|---|
| Effect of potassium | 1 | 31·0** | Interactions (*Continued*) | | |
| Effect of magnesium | 1 | 45·4** | Potassium × levels | 2 | 1·0 |
| Effect of calcium | 1 | 14·4** | Magnesium × levels | 2 | 0·1 |
| Effect of washing | 1 | 866·4** | Calcium × levels | 2 | 2·0 |
| Between levels | 2 | 0·2 | Potassium × ejaculate | 5 | 3·9** |
| Between ejaculates | 5 | 109·8** | Magnesium × ejaculate | 5 | 2·7* |
| Interactions | | | Calcium × ejaculate | 5 | 5·3** |
| Potassium × magnesium | 1 | 0·2 | Washing × ejaculate | 5 | 24·3** |
| Potassium × calcium | 1 | 1·2 | Levels × ejaculate | 10 | 0·8 |
| Magnesium × calcium | 1 | 8·0** | Levels × washing | 2 | 0·2 |
| Potassium × washing | 1 | 7·1** | Second-order interactions | 86 | 29·5† |
| Magnesium × washing | 1 | 1·1 | Higher-order interactions | 146 | 22·6 |
| Calcium × washing | 1 | 0·9 | | | |

* $P < 0·05$.        ** $P < 0·01$.

† The second-order interaction mean square is significantly larger than the higher-order interaction mean square ($F = 1·3$, $P = 0·05$) and has been used as error term.

to fowl spermatozoa by repeated washing is not due to the mechanical effect of centrifuging alone and is partly offset by the addition of potassium and magnesium.

### (b) Systematic Studies of Potassium, Magnesium, and Calcium

In view of the beneficial action of the diluent containing potassium and magnesium, especially after washing, more detailed factorial studies were made of these two ions and of calcium.

Potassium, magnesium, and calcium ions were added to the control diluent at levels 1, 2, and 3 (Table 1) so that within each level all possible combinations of the three ions were tried but no between-level combinations were made. In all diluents containing the highest concentration of calcium a slight opalescence was noted due to the precipitation of calcium phosphate.

Half of each pooled ejaculate was partitioned between the diluents to give a 1 in 10 dilution of semen. The other half was diluted 1 in 10 with control diluent and washed twice before partitioning. Motility was scored at 4, 8, 16, and 24 hr from the start of each test. The results for six replicates and the analysis of variance are presented in Table 3. The SILLIAC electronic computer was used for this analysis. As Bartlett's test showed that the variances of the second-order interactions were homogeneous ($\chi^2 = 20 \cdot 5$, 19 degrees of freedom, $P = 0 \cdot 3$), the pooled variance was used as error term since it was significantly greater than the higher-order interaction mean square.

Potassium, magnesium, and calcium all cause a highly significant increase in motility but the effects of magnesium are greater than those of potassium or calcium. Additive effects were given by potassium in combination with magnesium, calcium, or magnesium plus calcium but the motility in the diluent containing magnesium and calcium was significantly less than would be expected from the sum of their separate effects and was almost equal to the motility in magnesium alone. No significant variation in motility was seen between the three metal levels. As in the previous experiment, washing had a detrimental effect on motility and the beneficial action of potassium was greater on washed than on unwashed cells.

In the course of this experiment, marked agglutination of the spermatozoa was noted in tubes containing either level 2 or 3 of calcium. Thus in four ejaculates agglutination was found in eight of the 24 tubes containing calcium singly or in combination at level 2, and in 19 of the 24 tubes at level 3. Agglutination occurred as frequently in the unwashed as in the washed samples and neither potassium nor magnesium influenced the effect.

### (c) Potassium Toxicity

Further studies were undertaken to determine the toxic level of potassium for fowl spermatozoa. It was impossible to obtain toxic levels of magnesium and calcium since the phosphate buffer diluent was saturated with these ions at the highest level previously tried.

Completely replacing the sodium chloride in the control diluent with potassium chloride had no detrimental effect on motility. Toxic levels of potassium were, however, obtained by using potassium phosphate buffer in the diluent and then replacing the sodium chloride by increasing amounts of potassium chloride. The final concentrations of potassium in the three diluents prepared in this way were

150, 180, 210 mm. Tests with five ejaculates (Table 4) showed a linear fall in motility with increasing potassium concentrations.

### (d) Comparison of Alkali Metals

Since low concentrations of potassium increased the motility of fowl spermatozoa it was of interest to compare the effectiveness of the other alkali metals. The ammonium ion was also included in these tests as it substitutes for potassium in some biological systems (Boyer, Lardy, and Phillips 1942, 1943; Muntz 1947).

TABLE 4

EFFECT OF HIGH POTASSIUM CONCENTRATIONS ON THE MOTILITY OF FOWL SPERMATOZOA

| Potassium Concentration (mM) | Ejaculate | | | | | Totals |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| 0 | 62 | 60 | 63 | 62 | 64 | 311 |
| 150 | 58 | 59 | 62 | 61 | 62 | 302 |
| 180 | 56 | 56 | 49 | 54 | 57 | 272 |
| 210 | 49 | 45 | 43 | 50 | 41 | 228 |
| Totals | 225 | 220 | 217 | 227 | 224 | 1113 |

Potassium, lithium, rubidium, calcium, and ammonium chlorides were added to the control diluent to give a final concentration of 5 mM. Fowl spermatozoa were washed twice in the control diluent. The final volume of the suspension was adjusted to that of the original semen and aliquots were mixed with nine parts of the diluents containing the alkali metals. In order to produce a greater and more rapid effect, the diluted sperm suspensions were kept at 37°C for the first 2 hr before being scored at room temperature.

Motility was observed at 2, 4, 6, 8 hr from the start of each test and Table 5 shows the result for six replications. Potassium and rubidium significantly increased motility but the ammonium ion depressed it; lithium had no effect under these conditions (Table 5). If, however, the sperm suspensions were kept at room temperature throughout the test, motility in the control diluent was maintained better and it was possible to demonstrate a toxic effect of lithium.

### IV. DISCUSSION

Washing harms fowl spermatozoa to about the same extent as it does ram and bull spermatozoa (White 1953d), but they pack down into a particularly solid mass on centrifuging and are much more difficult to resuspend. This may account for the harmful effect of centrifuging fowl spermatozoa four times. Centrifuging cannot be the most important factor in washing, however, since the motility of centrifuged spermatozoa was much better than that of the washed cells, particularly at the start of experiments. As the beneficial effect of potassium was relatively greater

after washing, loss of this ion from the cell may account for some of the damage. The motility of spermatozoa washed in the potassium-containing diluents was, however, still below the centrifuged control and other substances such as cytochrome *c* and glyceraldehyde 3-phosphate dehydrogenase are probably also leached from the spermatozoa (Mann 1951; Smith, Mayer, and Merilan 1957).

TABLE 5

EFFECT OF ALKALI METAL IONS AND AMMONIUM IONS ON THE MOTILITY OF TWICE-WASHED FOWL SPERMATOZOA

| Ions Added | Ejaculate | | | | | | Totals |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| Nil | 26 | 19 | 10 | 17 | 54 | 20 | 146 |
| Potassium | 50 | 51 | 32 | 35 | 59 | 49 | 276 |
| Lithium | 14 | 16 | 11 | 18 | 44 | 15 | 118 |
| Rubidium | 37 | 39 | 30 | 40 | 56 | 49 | 251 |
| Caesium | 31 | 25 | 14 | 37 | 42 | 29 | 178 |
| Ammonium | 10 | 11 | 6 | 24 | 30 | 17 | 98 |

Analysis of Variance

| Source of Variation | Degrees of Freedom | | Variance Ratio | |
|---|---|---|---|---|
| Between ejaculates | 5 | | $16 \cdot 7$** | |
| Between diluents | 5 | | $24 \cdot 7$** | |
| Sodium *v.* potassium | | 1 | | $40 \cdot 2$** |
| Sodium *v.* lithium | | 1 | | $1 \cdot 9$ |
| Sodium *v.* rubidium | | 1 | | $26 \cdot 3$** |
| Sodium *v.* caesuim | | 1 | | $2 \cdot 4$ |
| Sodium *v.* ammonium | | 1 | | $5 \cdot 6$* |
| Residual | 25 | | 35 | |

\* $P < 0 \cdot 05$.          \*\* $P < 0 \cdot 01$.

These experiments clearly establish the importance of potassium and magnesium for the maintenance of full viability of fowl spermatozoa and in contrast to its action on ram and bull spermatozoa (Lardy and Phillips 1943, Blackshaw 1953b) calcium also proved beneficial. Diluents containing the lowest level of potassium and magnesium should prove quite satisfactory for the spermatozoa of this species. It is inadvisable to include calcium in such diluents since it causes agglutination in concentrations above $0 \cdot 3$ mM. Fowl spermatozoa, like those of the ram and bull (White 1953c) are very tolerant to high potassium levels but diluents composed entirely of potassium salts are undesirable.

Since the beneficial action of potassium was not influenced by magnesium or calcium it seems likely that the divalent ions act at a different site from potassium

in the spermatozoa. Magnesium and calcium, on the other hand, may have a similar role since the results suggest that they are interchangeable in their effect on motility. Similar results have been obtained by Wales and White (1958) using dog spermatozoa. Potassium and magnesium are important for several different reactions in the glycolytic cycle and may exert their stimulating action on fowl spermatozoa in this way.

The reaction of fowl spermatozoa to the rarer alkali metals is similar to that of the ram, bull, and dog (White 1953c; Wales and White 1958) and the ability of rubidium to replace potassium in spermatozoa would seem to be a widespread phenomenon. Lithium on the other hand appears to be generally spermicidal; it depresses the motility of human as well as ram, bull, and dog spermatozoa (MacLeod, Swan, and Aitken 1949; White 1953c; Wales and White 1958). Fowl spermatozoa are unique in that their motility is depressed by concentrations of ammonium ions innocuous to other species (White 1953c).

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# THE EXISTENCE AND STABILITY OF SELECTIVELY BALANCED POLYMORPHISM AT A SEX-LINKED LOCUS*

By J. H. Bennett†

*Summary*

Necessary and sufficient conditions are given for the existence and stability of a selectively balanced polymorphism at a sex-linked locus. It is shown that selective superiority of the heterozygote is neither necessary nor sufficient and also that the occurrence of heterozygotes with a frequency greater than that of homozygotes does not necessarily indicate that the heterozygote has a selective advantage over both homozygotes. These results are considered in relation to Wallace's published data for populations of *Drosophila pseudoobscura* in which selection is acting on the sex-linked condition "sex ratio".

## I. Introduction

In a large random mating population and in the absence of selective differences, the genotypes at an autosomal diploid locus with two allelomorphs $A$ and $a$ having population frequencies $p$ and $q$ respectively, where $p+q=1$, have the equilibrium population frequencies $p^2$ $AA$, $2pq$ $Aa$, and $q^2$ $aa$. In this case the frequency of heterozygotes ($2pq$) cannot exceed the total frequency of homozygotes ($p^2+q^2$). Heterozygotes and homozygotes are equally frequent only when $p=q=0\cdot5$. When there are genotypic selective differences, the same in the two sexes, a stable balanced polymorphism exists if, and only if, the heterozygote is at a selective advantage over both homozygotes. In this case the equilibrium population frequency of the heterozygote may exceed the total frequency of homozygotes. When there are differences between the sexes in respect of the genotypic selective values, the situation may be much more complex. Owen (1953) has shown, for example, that in this case there may be two distinct stable states of balanced polymorphism. Owen's demonstration emphasizes the necessity of recording observations for males and females separately when studying polymorphic populations. The conditions for stability have been determined recently for polymorphic situations dependent on a number of other more complex genetic systems, such as those involving three or more allelomorphs (Owen 1954; Kimura 1956$a$; Penrose, Smith, and Sprott 1956), a pair of autosomal loci (Kimura 1956$b$), and a sex-linked locus (Bennett 1957). It is with an elaboration of the last case that we are particularly concerned here.

## II. Polymorphic Equilibrium and the Conditions for Stability

In a large random mating population and in the absence of selective differences, the genotypes at a completely sex-linked locus with two allelomorphs $A$ and $a$

have the equilibrium frequencies $p^2$ $AA$, $2pq$ $Aa$, and $q^2$ $aa$ in the homogametic sex (which we shall suppose to be female) and $p$ $A$, $q$ $a$ in the heterogametic (male) sex, where $p$ and $q$ are the population frequencies of the genes $A$ and $a$ respectively. It has commonly been assumed that when there are selective differences between the genotypes, the same stability conditions apply to the genotypic selective values for the homogametic sex as to those values for an autosomal locus with no sex difference, i.e. the heterozygote has been assumed to be at a selective advantage over both homozygotes. Da Cunha (1953) has gone on to argue that when the population frequency of heterozygotes is greater than 50 per cent., then the heterozygote may be presumed to be at a selective advantage over both homozygotes. Other authors also (e.g. Birch and Battaglia 1957) have based claims for the selective superiority of heterozygotes for genes or structural arrangements in the $X$-chromosome in *Drosophila* populations upon an examination of data for females only. However, it is not enough to consider population data for just one sex. The frequencies and relative selective values of the different genotypes in both sexes must be taken into account (Bennett 1957).

Suppose that the selective values of the two male genotypes $A$ and $a$ are in the ratio of $t_A : 1$, where $t_A = 1 + h_A$, say, will be taken to be greater than 1, and that the selective values of the female genotypes $AA$, $Aa$, and $aa$ are in the ratio $S_{AA} : 1 : S_{aa}$. If $p_f$ and $q_f$, where $p_f + q_f = 1$, are the relative frequencies of the genes $A$ and $a$ in the gametic output of female members of a given generation, and $p_m$, $q_m$, where $p_m + q_m = 1$, are the corresponding frequencies for males, then with random mating, the genotypes will appear in the next generation with the following frequencies:

| | | | |
|---|---|---|---|
| Females | $p_f p_m$ $AA$ | $(p_f q_m + q_f p_m)$ $Aa$ | $q_f q_m$ $aa$ |
| Males | $p_f$ $A$ | $q_f$ $a$ | |

The relative frequencies of the genotypes in the same generation *after* selection will be as follows:

| | | | |
|---|---|---|---|
| Females | $S_{AA} p_f p_m$ $AA$ | $(p_f q_m + q_f p_m)$ $Aa$ | $S_{aa} q_f q_m$ $aa$ |
| Males | $t_A$ $p_f$ $A$ | $q_f$ $a$ | |

It is assumed that these are the frequencies with which the different genotypes contribute to the gametic output. When equilibrium is attained, the gametic output of females and males will show characteristic values for the gene ratio and these values will then be maintained throughout subsequent generations. The equilibrium values of the gene ratios in the gametic output of females and males are given by

$$u_f = \frac{p_f}{q_f} = \frac{2S_{AA}u_m u_f + (u_m + u_f)}{2S_{aa} + (u_m + u_f)},$$

and

$$u_m = \frac{p_m}{q_m} = t_A u_f.$$

It follows that

$$u_f = \frac{1 + t_A - 2S_{aa}}{1 + t_A - 2t_A \cdot S_{AA}}, \quad \dots\dots\dots\dots\dots\dots\dots (1)$$

and

$$u_m = t_A u_f. \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (2)$$

It can be shown that the gene ratios can have stable equilibrium values, other than zero or infinity, if, and only if, both numerator and denominator of the expression on the right hand side of equation (1) are positive, i.e. $S_{aa}$ must be less than $1 + \frac{1}{2}h_A$ and $S_{AA}$ must be less than $1 - [h_A/2(1+h_A)]$. In particular, when $t_A = 1$, i.e. when the gene substitution considered has no selective effect in males, the necessary and sufficient condition for the existence and stability of an equilibrium is that the heterozygote should be at a selective advantage over both homozygotes. Such a situation presumably exists only very rarely. Clearly, when $t_A$ has any value other than unity, it is neither necessary nor sufficient for the heterozygote to be at a selective advantage with respect to both homozygotes in order that a stable balanced polymorphism may exist. Thus, for example, when $t_A = 1 \cdot 5$ and $S_{AA} = 0 \cdot 8$, $S_{aa} = 1 \cdot 1$, there is a stable polymorphic equilibrium, but when $t_A = 1 \cdot 5$ and $S_{AA} = S_{aa} = 0 \cdot 9$, only a trivial equilibrium exists, the gene $a$ being eliminated from the population.

TABLE 1

OVERALL SELECTIVE VALUES GIVEN BY WALLACE (1948)

| Temp. (°C) | Selective Values | Males | | Females | | |
|---|---|---|---|---|---|---|
| | | $SR$ | $ST$ | $SR/SR$ | $SR/ST$ | $ST/ST$ |
| 16·5 | Maximum | 1·452 | 1 | 0·343 | 1 | 0·849 |
| 16·5 | Minimum | 1·393 | 1 | 0·278 | 1 | 0·734 |
| 25 | Maximum | 0·724 | 1 | 0·021 | 1 | 0·314 |
| 25 | Minimum | 0·696 | 1 | 0·014 | 1 | 0·277 |

### III. An Application to Wallace's Data on "Sex Ratio"

Wallace (1948) has reported extensive observations on selection acting on sex ratio ($SR$) and the normal $X$-chromosome ($ST$) in experimental populations of *D. pseudoobscura*. $SR$ males produce very few, if any, $Y$-bearing sperm (the actual proportion produced depending in part on the temperature), and have progenies consisting almost entirely of females. Since $SR$ causes the elimination of the $Y$-chromosome and an additional division of the $X$-chromosome during spermatogenesis, an $SR$ male contributes twice as many $X$-chromosomes to the population as does a normal male. However, selection acts to prevent $X$-chromosomes bearing $SR$ from replacing normal $X$-chromosomes throughout the population.

Wallace studied four different populations, two of which he maintained at 16·5°C and two at 25°C, and he found that in all cases the frequency of $SR$ declined from its initial high values. $SR$ was soon eliminated from the populations kept at 25°C but in those kept at 16·5°C it was reduced only to about 6 per cent. when, it seemed, equilibrium existed. Adaptive values for various phases of the life cycle and maximum and minimum overall selective values were published by

Wallace for these populations. The overall selective values which he gave are shown in Table 1. When these selective values are tested in the manner indicated above, it is found that they all correspond with stable non-trivial equilibria. The expected equilibrium values of the genotypic frequencies corresponding to these selective values are given in Table 2. These expected frequencies differ considerably from Wallace's observed frequencies and we may conclude that the true selective values are not within the ranges given by that author.

This finding leads us to question Wallace's methods of estimating selective values. We shall not enter upon an examination of these methods here but we may note in passing that when a non-trivial equilibrium exists (as Wallace says is the case for the populations maintained at $16 \cdot 5°$C) and the equilibrium genotypic frequencies are known, it may be possible by using the equations given above to obtain estimates of the genotypic selective values in terms of the observed equilibrium frequencies.

TABLE 2

EQUILIBRIUM VALUES FOR THE GENOTYPIC FREQUENCIES CORRESPONDING TO THE SELECTIVE
VALUES OF TABLE 1

| Temp. (°C) | Selective Values | Genotypic Frequencies | Males | | Females | | |
|---|---|---|---|---|---|---|---|
| | | | $SR$ | $ST$ | $SR/SR$ | $SR/ST$ | $ST/ST$ |
| $16 \cdot 5$ | Maximum | Before selection | $0 \cdot 341$ | $0 \cdot 659$ | $0 \cdot 146$ | $0 \cdot 478$ | $0 \cdot 376$ |
| | | After selection | $0 \cdot 429$ | $0 \cdot 571$ | $0 \cdot 059$ | $0 \cdot 564$ | $0 \cdot 377$ |
| | Minimum | Before selection | $0 \cdot 364$ | $0 \cdot 636$ | $0 \cdot 161$ | $0 \cdot 485$ | $0 \cdot 354$ |
| | | After selection | $0 \cdot 443$ | $0 \cdot 557$ | $0 \cdot 057$ | $0 \cdot 614$ | $0 \cdot 329$ |
| $25$ | Maximum | Before selection | $0 \cdot 393$ | $0 \cdot 607$ | $0 \cdot 125$ | $0 \cdot 461$ | $0 \cdot 413$ |
| | | After selection | $0 \cdot 319$ | $0 \cdot 681$ | $0 \cdot 004$ | $0 \cdot 777$ | $0 \cdot 219$ |
| | Minimum | Before selection | $0 \cdot 405$ | $0 \cdot 595$ | $0 \cdot 130$ | $0 \cdot 466$ | $0 \cdot 404$ |
| | | After selection | $0 \cdot 322$ | $0 \cdot 678$ | $0 \cdot 003$ | $0 \cdot 804$ | $0 \cdot 193$ |

## IV. EQUILIBRIA WITH AN EXCESS OF HETEROZYGOTES

Not only is a selective advantage of the heterozygote over both homozygotes in the homogametic sex neither necessary nor sufficient for there to exist a stable polymorphism, balanced by selection, at a sex-linked locus but also the occurrence of heterozygotes with a frequency greater than that of homozygotes does not necessarily indicate that the heterozygote has a selective advantage over both homozygotes. As an example of a population which, at equilibrium, has more heterozygotes than homozygotes but in which the heterozygote is not at a selective advantage over both homozygotes we may take the case where $t_A = 6$, $S_{AA} = \frac{1}{3}$, and $S_{aa} = 2$; 64 per cent. of adult females in such a population will have the genotype $Aa$ when equilibrium is attained.

Populations with polymorphisms dependent on sex-linked loci are of great genetical interest because of the fact that such polymorphic situations can be maintained by selection with or without selective superiority of the heterozygote. Their further study, both in the field and the laboratory, may therefore be expected to give valuable information on the part played by heterosis in maintaining polymorphism.

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# SIMULATION OF GENETIC SYSTEMS BY AUTOMATIC DIGITAL COMPUTERS

## III. SELECTION BETWEEN ALLELES AT AN AUTOSOMAL LOCUS

### By J. S. F. BARKER*

### Summary

A new approach to analysis of the effect of selection on gene frequencies is described. An electronic digital computer (the SILLIAC) is used to simulate the selection processes that operate in populations. The Monte Carlo method permits inclusion of stochastic processes so that results should simulate those in natural or experimental populations.

The programme simulates selection between two autosomal alleles and allows for selection at four stages of the life cycle, viz.:

   (i) Zygote selection: ability to survive from fertilization to sexual maturity.

   (ii) Reproductive selection: differential reproductive ability of different genotypes.

   (iii) Selection between gametes at meiosis in heterozygotes.

   (iv) Selection between gametes on their ability to take part in fertilization.

These selective values and population size can be varied to investigate different genetic situations.

To test the programme, two experiments reported in the literature have been simulated, viz.:

   (i) Selection between *ST* and *CH* chromosomal arrangements in the third chromosome of *Drosophila pseudoobscura* (Dobzhansky and Pavlovsky 1953).

   (ii) Selection between *glass* and wild type in *Drosophila melanogaster* (Merrell and Underhill 1956).

Close agreement was obtained with the results of Dobzhansky and Pavlovsky. The importance of an adequate estimate of generation length in making these comparisons is discussed. Agreement with the results of Merrell and Underhill was not so close. Possible reasons for this in terms of selective mating are discussed.

The results show that it is possible to simulate selection between two alleles at an autosomal locus by using automatic digital computers.

## I. INTRODUCTION

In this paper, a new approach to the analysis of selection is described. Insofar as certain parameters must be specified at the beginning of the analysis, the results are restricted. However, as these parameters may be varied at will, the extent to which the analysis may be taken as general can be arrived at by repeated analysis with different basic parameters. In this way, the extent of the generality of the conclusions and the particular factors of most importance in determining the results are revealed.

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The method simulates the processes which go on in a population and calculates in an electronic digital computer (the SILLIAC) the effects which these processes will have on gene frequency. The method is basically that described by Fraser (1957$a$, 1957$b$) in which the Monte Carlo method has been adapted for genetic analysis of populations. This method does not involve the use of complex mathematics common to this field. In their place, long series of simple arithmetic steps are performed in an electronic computer. One feature of this approach is that the calculations follow sequences closely related to the life cycles of animals, and are therefore much easier to understand than more subtle methods. An important feature of this "arithmetic" method is the inclusion of "stochastic simulants", i.e. arithmetic processes which introduce random variation into the main sequence. For a specified set of population parameters, inclusion of a different "stochastic simulant" for each of a number of runs means that each series of calculations will produce a different answer. Thus a number of such answers can be combined to give both the mean and the variance of the processes under investigation.

In the analysis discussed here, one autosomal gene with two alleles $A$ and $a$ is segregating in a bisexual population in which there is no overlap of generations. A programme has been written which sets the SILLIAC to simulate this genetic model, allowing for selection between the two alleles at different stages of the life cycle. The various selection coefficients and the population size can be varied to investigate different genetic situations. In this paper, the operations of the programme are described and the validity of the simulation tested.

## II. Selection Processes

The numbers of fertilized zygotes are taken as the reference point for the beginning of each generation. The selection processes which go on in the population are as follows:

(i) The first selection is on the ability of genotypes to survive from fertilization to sexual maturity. This may be different in the two sexes which are therefore treated separately. It is assumed that each individual of a given genotype has a certain probability of surviving to sexual maturity. However, there is a chance element in this survival. Parents are selected, not in exact proportions according to the probability of survival, which is the mean result in an infinite population, but according to the operation of a random or chance effect on the probability. The number of parents so selected is calculated.

(ii) Once the parents are selected, they must generate gametes. Selection operates again in that certain females may be more fecund than others, or more readily mated, or more long-lived. In the male, some may be more aggressive at getting mates, or more long-lived. Again, the probability of a male or female producing gametes which take part in fertilization is specified and the gametes picked out by the operation of a chance effect on these probabilities, rather than as clear-cut proportions.

(iii) In the formation of gametes in heterozygotes, they may not be produced in a 1 : 1 ratio. Selection of this type may be specified and the number of $A$

and $a$ gametes determined, again with the introduction of the chance effect. For example, "sex ratio" in *Drosophila pseudoobscura* (Sturtevant and Dobzhansky 1936) is of this type.

(iv) Once the gametes are formed, selection again may operate to determine what proportion of gametes of each kind are successful, for example, the $S$ genes in plants (Bateman 1952).

It should be noted that if it is specified that there is no selection of types (ii), (iii), and (iv) above, then type (i) can be used as relative adaptive values, specifying overall selection.

TABLE 1

INFORMATION REQUIRED ABOUT ZYGOTES

| Genotype | No. of Females | Zygote Selective Value | Genotypic Reproductive Coefficient | No. of Males | Zygote Selective Value | Genotypic Reproductive Coefficient |
|---|---|---|---|---|---|---|
| *AA* | $N_{f_1}$ | $w_f$ | $x_{f_1}$ | $N_{m_1}$ | $w_m$ | $x_{m_1}$ |
| *Aa* | $N_{f_2}$ | $w_f(1-h_f s_f)$ | $x_{f_2}$ | $N_{m_2}$ | $w_m(1-h_m s_m)$ | $x_{m_2}$ |
| *aa* | $N_{f_3}$ | $w_f(1-s_f)$ | $x_{f_3}$ | $N_{m_3}$ | $w_m(1-s_m)$ | $x_{m_3}$ |
| Totals | $N_f$ | | 1 | $N_m$ | | 1 |

## III. Operations of the Programme

At the start, the alleles $A$ and $a$ will be distributed with a certain frequency in the population and will be present in zygotes $AA$, $Aa$, and $aa$, the frequency of which must be specified. The starting point is taken as the moment of fertilization. The information required about zygotes can be tabulated as in Table 1. The intensity of selection is $s$; introducing $h$ makes the formulae general for all degrees of dominance (Lush 1948). With this information it is possible to take the population to the point where gametes are produced.

The first step is to determine the number of females which reach sexual maturity. To do this $N_{f_1}$, $N_{f_2}$, and $N_{f_3}$ are multiplied by the appropriate zygote selective values to give the expected number of females surviving to sexual maturity, viz. $N'_{f_1}$, $N'_{f_2}$, and $N'_{f_3}$, whose sum is $N'_f$. The actual number will vary in a random way about the expected. The random choice is put in by generating a random number $v_i$, which lies between 0 and 1. This random number $v_i$ is multiplied by $N'_f$ and the product will lie somewhere between 0 and $N'_f$. If it lies between 0 and $N'_{f_1}$, it is taken to represent an $AA$ individual, if it lies between $N'_{f_1}$ and $N'_{f_1} + N'_{f_2}$ it is taken to be an $Aa$ individual, and if it lies between $N'_{f_1} + N'_{f_2}$ and $N'_f$, it is taken to be an $aa$ individual. This process is repeated with $N'_f$ random numbers so that $N'_f$ individuals are generated and classified as $AA$, $Aa$, and $aa$

in such a way that the mean proportions in an infinite population will be $N'_{f_1} : N'_{f_2} : N'_{f_3}$, but in a finite population will deviate from these values by chance. This is repeated for males and we now have the numbers of females and males at sexual maturity as follows:

| Genotype | No. of Females | No. of Males |
|----------|----------------|--------------|
| $AA$ | $N_{F_1}$ | $N_{M_1}$ |
| $Aa$ | $N_{F_2}$ | $N_{M_2}$ |
| $aa$ | $N_{F_3}$ | $N_{M_3}$ |
| Totals | $N'_f$ | $N'_m$ |

At this stage, a device is introduced to prevent the population increasing or decreasing indefinitely. The expected size $(E)$ of the population is specified and the average number of offspring of each female is made to be $E/N'_f$ as will be shown later. Thus the size of the population produced by females will tend towards $E$ but will fluctuate around it according to chance. The relative reproductive abilities of different genotypes must also be introduced. Taking females first, let the average number of offspring of each genotype be $P_1$ for $AA$, $P_2$ for $Aa$, and $P_3$ for $aa$, so that

$$\tfrac{1}{3}(P_1 + P_2 + P_3) = E/N'_f.$$

As

$$x_{f_1} + x_{f_2} + x_{f_3} = 1,$$

$$3E/N'_f \times x_{f_1} = P_1,$$

$$3E/N'_f \times x_{f_2} = P_2,$$

$$3E/N'_f \times x_{f_3} = P_3,$$

$P_1$, $P_2$, and $P_3$ may thus be calculated.

We now have $N_{F_1}$ $AA$ females tending to produce an average of $P_1$ offspring each, $N_{F_2}$ $Aa$ tending to produce $P_2$, and $N_{F_3}$ $aa$ tending to produce $P_3$. The actual numbers are formed by a further random process in which $P_i$ is assumed to be the mean of a Poisson distribution. This is an ideal situation and, as Crow (1954) shows, will be most accurate where $P_i = 2$. By calculating $e^{-P_1}$ and multiplying together a series of random numbers $v_0, v_1, \ldots \ldots v_n$ until the product is less than $e^{-P_1}$, a random Poisson deviate $n$ is formed which is taken to be the actual number of offspring produced by a particular $AA$ female. This process is repeated $N_{F_1}$ times for $AA$ females. Using $e^{-P_2}$, it is repeated $N_{F_2}$ times for $Aa$ females, and using $e^{-P_3}$, $N_{F_3}$ times for $aa$ females. We now have the number of gametes from each female which take part in fertilization. They still have to be sorted into $A$ and $a$. All those produced by $AA$ females will be $A$; call the total number of these $F'_1$. All those from $aa$ females will be $a$; call the total number of these $F'_3$. Call the total number from $Aa$ females $F'_2$. If there is selection between

gametes at meiosis in heterozygotes with selection coefficient of $A$ equal to $r_{f_1}$, and of $a$ equal to $r_{f_2}$, the expected proportions of $A$ and $a$ gametes will be

$$F_2'/2 \times r_{f_1} : F_2'/2 \times r_{f_2}.$$

Use of the same operation as was used before to choose the actual number of females of each genotype surviving to sexual maturity—in this case operating on the above expected proportions—makes the choice of $A$ and $a$ random. As $F_2'$ has been determined as the number of gametes from $Aa$ females, this random transform must generate a total of $F_2'$ $A$ and $a$ gametes. By adding the numbers of $A$ and $a$ gametes we get the total of $A$ and $a$ gametes produced by females.

There is one further step to take: selection between gametes at fertilization. This is done by a random transform as before. The total number of gametes produced by females and that will take part in fertilization are made to equal $F_1' + F_2' + F_3'$, which is called $F'$.

These processes are repeated for males to get the numbers of male $A$ and $a$ gametes that will take part in fertilization, where the total is $M'$. The processes are not such that $F'$ necessarily equals $M'$, while, for fertilization, there must be equal numbers of male and female gametes. However, they are not likely to be very different and the number of offspring generated is set to equal $F'$ or $M'$, whichever is the smaller.

Fertilization is then performed by random combination of gametes. One male and one female gamete are taken and the combination tested to see if it is $AA$, $Aa$, or $aa$. Another pair is taken, and so on. As this is done, the numbers of male and female $A$ and $a$ gametes are counted as they are used and tested against the numbers available for fertilization, until all the gametes of each type from each sex are used. Each offspring individual, once its genotype is determined, is operated on by a random transform on an expected $1:1$ ratio to determine if it is to be male or female.

The gene frequencies in males, in females, and overall are calculated from the total numbers of males and females of each genotype so generated in the offspring generation. The programme then prints out this information on the numbers of males and females of each genotype and the gene frequencies. It then uses these to produce the following generation, and so on. The programme can be set to run for any number of generations.

## IV. Results

The programme has been tested by trying to simulate experiments that are reported in the literature. Attempts have been made in some of these experiments to determine the nature and extent of the selection forces operating. These estimated selection coefficients can be used as specifications for the programme and the results of the simulated populations compared with those of the selection experiments. Two such experiments have been simulated, one with a large population, the other with a small.

## (a) *Large Population Size*

Using population cages, Dobzhansky and Pavlovsky (1953) studied com-
petition between the *ST* and *CH* chromosomal arrangements of *Drosophila pseudo-
obscura* in four populations. From their results, they calculated that the adaptive
values of the genotypes were: *ST/ST* 0·895, *ST/CH* 1·000, and *CH/CH* 0·413.
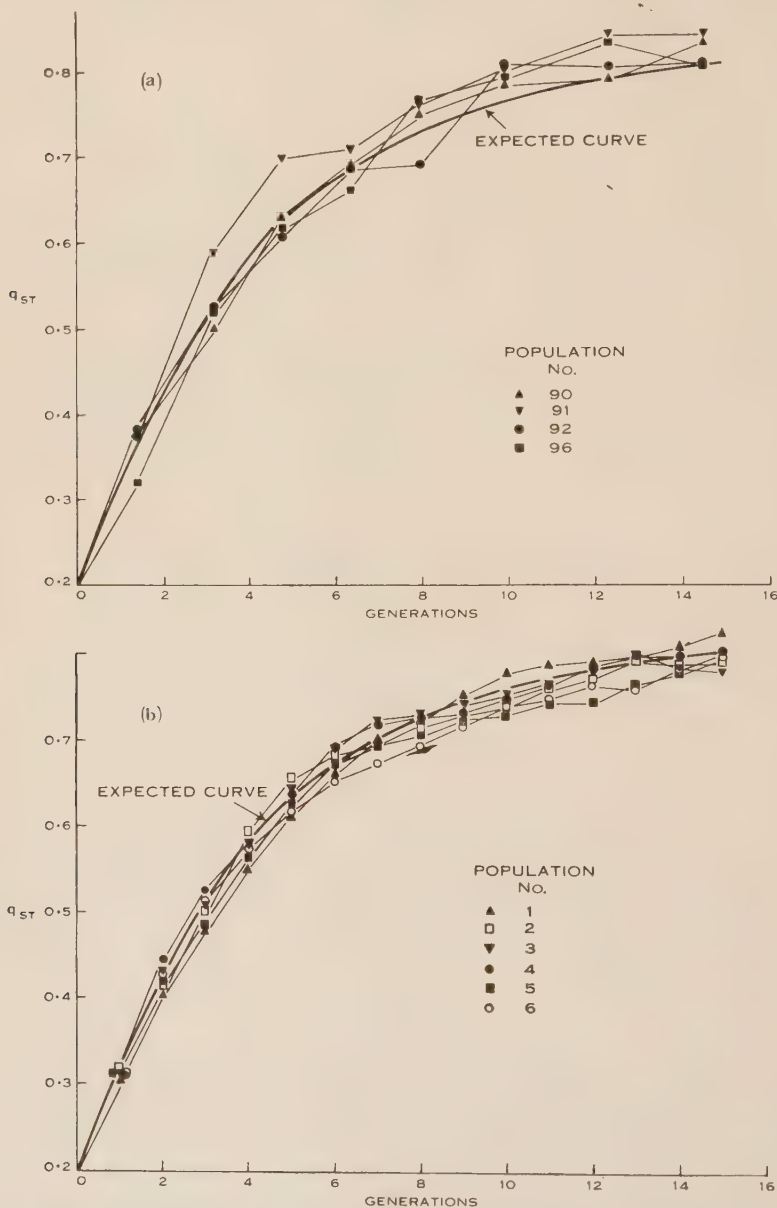


Fig. 1.—Frequencies of chromosomes with the *ST* gene arrangement. (*a*) Results of Dobzhansky
and Pavlovsky's (1953) four experimental populations. (*b*) Results of six simulated populations.
The smooth solid curve in both sets is the expected curve with adaptive values of *ST/ST* :
*ST/CH* : *CH/CH* genotypes being 0·895 : 1 : 0·413 respectively.

These adaptive values have been used as relative zygote selective values for both males and females. It was specified that there was no genotypic reproductive selection and no gamete selection. However, chance effects still operate at these stages in the cycle. $E$ was specified at 4000. Six replicate runs were made with these specifications. The results are shown in Figure 1. The generation length of the experimental population was assumed to be 25 days (Dobzhansky and Pavlovsky 1953).

## (b) Small Population Size

Merrell and Underhill (1956) using population bottles, studied changes in gene frequency in various mutants of *D. melanogaster* when in competition with their wild-type alleles. The changes in frequency were considered to be mainly a function of selective mating.

TABLE 2

RESULTS OF FEMALE-CHOICE MATING TESTS

Results between *glass* (*gl*) and wild-type flies (quoted from Merrell and Underhill 1956)

| Female Genotype | Successful Male (%) | |
| --- | --- | --- |
| | +/+ | *gl/gl* |
| +/*gl* | 96·3 | 3·7 |
| *gl/gl* | 100·0 | 0·0 |

The competition between *glass* (*gl*) and wild type has been simulated. Relative zygote selective values for both males and females have been taken as: +/+ 1·000, +/*gl* 1·000, and *gl/gl* 0·908, as given by Merrell and Underhill. These are not calculated on the basis of given values of $h$ and $s$ but are overall values determined experimentally. They quote also the results of female-choice mating tests which are given in Table 2.

From these, relative genotypic reproductive coefficients for males have been taken as: +/+ 0·48, +/*gl* 0·48, and *gl/gl* 0·04.

As there are no data on the occurrence or extent of selective mating by males when given a choice of females, the coefficients for females must be assumed to be identical for each genotype. The female coefficients are therefore: +/+ 0·33, +/*gl* 0·33, and *gl/gl* 0·33. No other selection was involved in the simulated populations and $E$ was specified as 180. The results are shown in Figure 2. The curve shown for the experimental populations is the average of nine populations as given by Merrell and Underhill. Generation length in these populations has been taken as 24 days (Merrell 1953).

## V. Discussion

### (a) Large Population Size

It will be noted from Figure 1 that four of the six simulated populations (Nos. 3, 4, 5, and 6) have identical frequencies of *ST* in generation 1. This results from an inherent difficulty in the programme. Dobzansky and Pavlovsky's populations were started with 600 *ST/CH* individuals and 900 *CH/CH*. With the given adaptive values and assuming no random processes, all the heterozygotes and 372 of the *CH/CH* individuals will contribute gametes to form generation 1. In the population cage, these flies will give rise to a population of at least 4000 in generation 1. However, in the simulated populations with a Poisson distribution of progeny numbers,
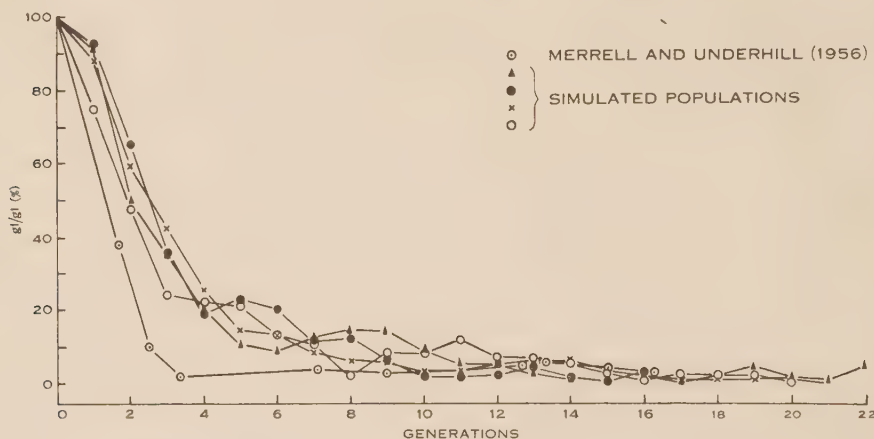


Fig. 2.—Results of competition between wild type and *glass*. ⊙ Average of the nine experimental populations of Merrell and Underhill (1956). ▲, ●, ×, ○ Simulated populations.

it takes the population two or three generations to reach equilibrium size. Therefore, the expected numbers of each genotype in generation 1 with a population size of 4000 have been calculated and these four simulated populations started from this point. The first two runs (simulated populations Nos. 1 and 2) were done using the numbers of each genotype as used by Dobzhansky and Pavlovsky as the starting point in generation 0.

Figure 1 shows that the results of the experimental and simulated populations agree fairly closely. One difference is the relative lack of generation to generation variation in the simulated compared to the experimental populations. This may be due to:

(i) In the simulated populations, generations are discrete, i.e., there is no generation overlap.

(ii) The variation in the experimental populations will include sampling variations, as each point is based on a sample of 300 chromosomes.

(iii) The adaptive values in the simulated populations are constant. In experimental populations, they would probably show some variation from generation to generation. This would increase generation to generation variation in the experimental populations.

It appears that overlapping generations have little effect on the results (except for the variation effects noted above). The question of actual generation length is important in this connection. Dobzhansky and Pavlovsky's adaptive value estimates depend on the assumption of a generation length of 25 days. If this is incorrect, the adaptive values are also incorrect. There is a suggestion from their results that they may be slightly incorrect. In their Figure 2, all except one of the results lie within the 95 per cent. confidence limits for the expected values of *ST* frequency. However, the estimates at 80, 120, and 160 days lie mainly below the expected curve but with the mean biased upwards by population 91, while those at 250, 310, and 365 days lie mainly above the expected curve. Therefore, assuming a slightly different generation length may give a better fit to the data. This question of generation length in population models has been discussed briefly by Moree (1955).

## (b) Small Population Size

The rate of change in percentage of *gl/gl* from the first to the third generation is similar in the simulated and experimental populations, but slightly lower in the former. It may be that *glass* females are not quite as efficient as wild type in mating, whereas they may have been assumed the same in the simulated populations.

In the first generation, however, and from the third to the twelfth, the curves show different trends. The lag (i.e. slower change) in the simulated populations in the first generation is difficult to explain. The experimental populations were started by placing one wild-type male into an established *glass* population. The immediate decrease in *gl/gl* frequency is surprising, as one would not expect this one male to be so successful in mating when so many *gl/gl* males are present. However, the first estimate in the experimental populations is at 30 days, and would include both first and second generation flies. Thus, the lag in the first generation may be real, but followed by a larger decrease in *gl/gl* frequency in the second generation than is shown by the simulated populations. This could operate through selective mating against *gl/gl* females. Some factor such as this, increasing the selection against *glass*, must be operating.

This is shown by the fact that seven runs were made in the SILLIAC but three of these went to 100 per cent. *glass* in the first generation. Merrell and Underhill (1956) state that, of their 10 experimental populations, only one went to 100 per cent. *glass*.

The slower change in the simulated populations from the third to the twelfth generation could be explained by the fact that, in these populations, the genotypic reproductive coefficients are constant throughout. It seems likely that as the frequency of *glass* males decreases, their mating success relative to wild type will also decrease. The number of females available to the *glass* males will be the important factor. *glass* males have a lower mating ability than wild type so that with a higher frequency of wild-type males than *glass* males in the population, the former would cover most of the females. This would result in an increase in the rate of change in *gl/gl* frequency.

The results of the two comparisons made above show that it is possible to simulate in an automatic digital computer the operations of selection between two alleles at an autosomal locus.

This programme will be used to generate families of curves relating changes of gene frequency of two autosomal alleles in competition to the parameters of population size and nature and intensity of selection.

## VI. ACKNOWLEDGMENTS

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# SIMULATION OF GENETIC SYSTEMS BY AUTOMATIC DIGITAL COMPUTERS

## IV. SELECTION BETWEEN ALLELES AT A SEX-LINKED LOCUS

### By J. S. F. BARKER*

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*Summary*

A programme simulating selection between two alleles at a sex-linked locus has been developed for an automatic digital computer (the SILLIAC). It introduces selection and chance effects at four stages of the life cycle.

Two experiments on competition between sex-linked alleles have been simulated, viz. selection between (i) the "sex ratio" and standard $X$-chromosomes of *Drosophila pseudoobscura* (Wallace 1948) and (ii) *yellow* and its wild-type allele in *D. melanogaster* (Merrell and Underhill 1956).

(i) Wallace's data includes estimated overall selective values and selective values for various of the components of fitness. From the latter, specific selective values appropriate to the four stages of selection in the programme have been calculated. Two sets of simulated populations, one using the overall selective values, the other the specific, gave similar results. Further, though completely dissimilar to the experimental population, they were very similar to the theoretical curve of change in frequency of sex ratio. The difficulties of estimating selective values from studies of the components of fitness are discussed and the need for an overall measure of fitness emphasized.

(ii) The simulated populations for competition between *yellow* and wild type agreed closely with the experimental. The importance of selective mating in this competition has been considered.

The results show that it is possible to simulate the operations of selection between two alleles at a sex-linked locus.

## I. INTRODUCTION

Changes in gene frequency due to differential selection of alleles at a sex-linked locus have been reported by Buzzati-Traverso (1955), L'Héritier and Tiessier (1934, 1937), Ludwin (1951), Merrell (1953a, 1953b), Merrell and Underhill (1956), Reed and Reed (1948, 1950), and Wallace (1948). Of these, Merrell, Reed and Reed, and Wallace have attempted to determine the selective forces operating in their populations. In most cases, however, one can only note that there is a fitness differential between the alleles but cannot determine the nature or extent of this differential.

Fraser (1957a, 1957b) first introduced the Monte Carlo method to the simulation of genetic systems by automatic digital computers. The method was used

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by Barker (1958) in the analysis of selection between alleles in a population where, for given selection coefficients, it is possible to simulate the operations of selection and generate selection curves. The results showed that the simulated selection curves, which involve chance effects at the various stages of selection, closely resemble the selection curves obtained in experimental populations. Selection between alleles at an autosomal locus was discussed.

This method has now been adapted to the study of selection between alleles at a sex-linked locus. The programme for the SILLIAC simulates selection between two alleles segregating in a bisexual population in which males are the heterogametic sex and there is no overlap of generations.

TABLE 1

INFORMATION CONCERNING ZYGOTES

| Genotype | No. of Males | Zygote Selective Value | Genotypic Reproductive Coefficient |
|---|---|---|---|
| $A/Y$ | $N_{m_1}$ | $w_m$ | $x_{m_1}$ |
| $a/Y$ | $N_{m_2}$ | $w_m(1-s_m)$ | $x_{m_2}$ |
| Totals | $N_m$ | | 1 |

## II. Operations of the Programme

The selection processes are as defined by Barker (1958) for selection between autosomal alleles, viz.:

(i) Zygote selection;

(ii) Genotypic reproductive selection;

(iii) Selection between gametes at meiosis in heterozygotes;

(iv) Gamete selection at fertilization.

Essentially, the operations of this programme are the same as those of the autosomal selection programme. The operations for females are identical but they differ slightly for males as there are only two male genotypes. The operations of selection will be considered briefly for males only. Designate the segregating alleles as $A$ and $a$. At the start, which is taken as the moment of fertilization, the numbers of $A/Y$ and $a/Y$ males are specified ($Y$ representing the $Y$-chromosome). The information required about zygotes is given in Table 1.

To determine the numbers of males which reach sexual maturity, $N_{m_1}$ and $N_{m_2}$ are multiplied by the appropriate zygote selective values to give the expected numbers of males. Chance effects are introduced as before (Barker 1958) to determine the actual numbers of males at sexual maturity, viz. $N'_{m_1}$ of $A/Y$ and $N'_{m_2}$ of $a/Y$, with a total of $N'_m$.

At this stage, a device is introduced which allows population size to tend to the specified expected size ($E$), but letting it fluctuate around this according to chance. The average number of offspring of each male is made to be $E/N'_m$. The relative genotypic reproductive coefficients are also effective at this stage. Let the average numbers of offspring of the two genotypes be $P_1$ and $P_2$ so that

$$(P_1+P_2)/2 = E/N'_m.$$

As

$$x_{m_1}+x_{m_2} = 1,$$

$$2E/N'_m \times x_{m_1} = P_1,$$

and

$$2E/N'_m \times x_{m_2} = P_2.$$

From each genotype the numbers of gametes which take part in fertilization are generated as before (Barker 1958). This gives the total numbers of gametes from $A/Y$ males (designated $M_1$) and from $a/Y$ males (designated $M_2$). These have to be sorted into gametes containing $A$, $a$, or $Y$. Take, for example, $A/Y$ males. If there is selection between the gametes at meiosis with selection coefficients of $A$ equal to $r_{m_1}$, and of $Y$ equal to $r_{m_3}$, the expected proportions of $A$ and $Y$ gametes will be $M_1/2 \times r_{m_1} : M_1/2 \times r_{m_3}$. Use of the random transform then generates the actual numbers of $A$ and $Y$ gametes produced by $A/Y$ males, viz. $M'_A$ of $A$, $M'_{YA}$ of $Y$.

Similarly for $a/Y$ males, where the selection coefficient of $a$ is $r_{m_2}$, the numbers of $a$ and $Y$ gametes are generated, viz. $M_a'$ of $a$, $M'_{Ya}$ of $Y$.

Selection between gametes at fertilization is then introduced, where the selective values are $t_{m_1}$ for $A$, $t_{m_2}$ for $a$, and $t_{m_3}$ for $Y$. The procedure is as above for selection between gametes at meiosis. Thus the actual numbers of gametes of each type that take part in fertilization are generated, namely $M_A$ of $A$, $M_{YA}$ of $Y$ (from $A/Y$ males), where $M_A+M_{YA} = M_1$; $M_a$ of $a$, $M_{Ya}$ of $Y$ (from $a/Y$ males), where $M_a+M_{Ya} = M_2$; and $M_1+M_2 = M'$, the total number of male gametes generated.

The numbers of gametes produced by females are $F_A$ of $A$, $F_a$ of $a$, where $F_A+F_a = F'$.

The processes are not such that $F'$ necessarily equals $M'$, while for fertilization, there must be equal numbers of male and female gametes. However, they are not likely to be very different, and the number of offspring generated is set to equal $F'$ or $M'$, whichever is the smaller.

Fertilization is performed in two stages, though in each stage the method is the same as that in the autosomal selection programme. First, fertilization by gametes from $A/Y$ males is carried out. This gives the following progeny genotypes: males, $A/Y$, $a/Y$; females, $AA$, $Aa$.

The numbers of each of these are counted as they are produced. When this is completed, the remaining female gametes are fertilized by gametes from $a/Y$ males, giving the following genotypes: males, $A/Y$, $a/Y$; females, $Aa$, $aa$.

This method is not entirely satisfactory for when $F'$ is less than $M'$, some gametes from $a/Y$ males will not be used in fertilization, while all those from $A/Y$ males will be used. There is then a possibility of bias in the results, the degree of this bias depending on how often $F'$ is less than $M'$, and on the extent of the difference between them. Although this bias is quite small, the programme has since been altered to remove this deficiency. This has been done by using a total of $F'$ male gametes when $F'$ is less than $M'$. Gametes from $A/Y$ and $a/Y$ males are used in proportion to the numbers of each previously generated. The possible degree of bias in the results due to the fertilization method used can be seen in the following. One of the simulated populations to be discussed later has been re-run for three generations using the amended programme. With the original programme, the gene frequencies were:

| Generation | Males | Females | Overall |
|------------|-------|---------|---------|
| 1 | 0·513 | 0·496 | 0·504 |
| 2 | 0·423 | 0·418 | 0·420 |
| 3 | 0·411 | 0·370 | 0·391 |

while the amended programme gave:

| Generation | Males | Females | Overall |
|------------|-------|---------|---------|
| 1 | 0·526 | 0·487 | 0·506 |
| 2 | 0·416 | 0·425 | 0·421 |
| 3 | 0·401 | 0·371 | 0·386 |

Another simulated population gave results with the amended programme identical to those previously obtained; in this case $F'$ was not less than $M'$ in any generation. There will be no bias when $M'$ is less than $F'$ (i.e. when there are unused female gametes), because female $A$ and $a$ gametes are used in a random order.

From the results of the two stages of fertilization, the total numbers of progeny of each of the five genotypes are obtained and then the gene frequencies of $A$ in males, females, and overall are calculated. The programme prints out this information on the number of progeny individuals of each genotype and gene frequencies. These are used to produce the following generation, and so on. The programme can be set to run for any number of generations.

The time taken by the SILLIAC to do these computations may be of interest. At an expected population size ($E$) of 180, 17 generations took 9 min, while when $E$ equalled 4000, 10 generations took 22 min.

## III. Results and Discussion

The aim so far has been to show that this porgramme can simulate the processes of selection between sex-linked alleles. Merrell (1953a, 1953b), Merrell and Underhill (1956), Reed and Reed (1950), and Wallace (1948) have estimated the nature and extent of the selective forces operating in their experimental populations. These estimated selection coefficients can be used as specifications for the programme, allowing the results of the simulated populations to be compared with those of the experimental populations. Two such experiments have been simulated.

As in the case of the autosomal selection, experiments using different population sizes have been selected, viz.: selection studied in large populations by Wallace (1948) and in small populations by Merrell and Underhill (1956).

### (a) Large Population Size

Wallace made an extensive analysis of selection between the "sex ratio" and standard $X$-chromosomes of $D.$ $pseudoobscura$, studying competition between these chromosomes in population cages of the type described by Wright and Dobzhansky (1946), which at 25°C support a population of about 4000 adults. In addition, he analysed selective differences by studying larval competition, adult longevity, fecundity, sexual activity, and egg hatchability. From these data, estimates of the selective values of the genotypes were made.

From Wallace's Table 16 (which summarizes the selective values he determined) specific selective values appropriate to the four stages of selection in the programme were calculated as follows for selection at 25°C.

### (i) Zygote Selective Values Used

(1) *Males.*—Selective values for larval competition.

(2) *Females.*—Larval competition $\times$ egg hatchability.

### (ii) Genotypic Reproductive Coefficients Used

(1) *Males.*—Longevity $\times$ sexual activity—maximum estimate of latter.

(2) *Females.*—Longevity $\times$ fecundity—maximum estimate of latter.

These values were then adjusted so that coefficients for males and females both sum to 1.

### (iii) Selection between Gametes at Meiosis in Heterozygotes

(1) *Males.*—Darlington and Dobzhansky (1942) found that at 25°C, males comprise 6·2 per cent. of the total offspring of sex ratio males. Therefore the selective values of gametes from sex ratio males would be 0·938 for sex ratio ($SR$) $X$-chromosomes, and 0·062 for $Y$-chromosomes, these values being in the ratio 1 : 0·066. However, in setting up the programme specifications, this ratio was taken inadvertently as 1 : 0·1. The effect of this will be noted later. The selective values were thus specified as 1·0 for $SR$ and 0·1 for $Y$. As there is no known fitness difference between the standard $X$-chromosome and the $Y$, the selective value for $ST$ was taken also as 0·1. In this case, there is no direct comparison between these fitnesses of sex ratio and standard, both are relative to the $Y$. This may be seen by considering the programme operations at this stage. The total number of gametes produced by $ST/Y$ males is determined and these are then apportioned according to the selective values into the numbers of $ST$ and $Y$. As the selective values are equal, this subdivision is essentially on a 1 : 1 expectation. Therefore, the selective values operate as 1·0 for $SR$ and 0·1 for $Y$ in sex ratio males, and 0·5 for $ST$ and 0·5 for $Y$ in standard males, giving a ratio of 2 : 1 for the selective values of $SR$ : $ST$. This is the ratio expected from the results of Sturtevant and Dobzhansky (1936). They showed that the $Y$-chromosome fails to be included in the spindle at the meiotic divisions

during spermatogenesis and is lost. The $X$-chromosome, which shows a four-parted structure at the first metaphase, undergoes two equational divisions during the course of meiosis resulting in the formation of four sperm, each containing a sex ratio $X$-chromosome. Thus a male of this type will produce only (or mainly) female offspring but these will be as numerous as the combined sexes in progeny of males with the normal $X$-chromosome.

(2) *Females.*—No differential selection so that coefficients are taken as $1 \cdot 0$.

(iv) *Gamete Selective Values*

No differential selection so that coefficients are taken as $1 \cdot 0$.

TABLE 2

MAXIMUM SELECTIVE VALUES AT 25°C FOR COMPETITION BETWEEN
SEX RATIO ($SR$) AND STANDARD ($ST$)

Values obtained from data of Wallace (1948)

| Selective Values | Males | | Females | | |
|---|---|---|---|---|---|
| | $SR$ | $ST$ | $SR/SR$ | $SR/ST$ | $ST/ST$ |
| Overall (fitnesses) | $0 \cdot 724$ | 1 | $0 \cdot 021$ | 1 | $0 \cdot 314$ |
| Specific | | | | | |
|   Zygote | $0 \cdot 407$ | 1 | $0 \cdot 036$ | 1 | $0 \cdot 511$ |
|   Genotypic reproductive coefficients | $0 \cdot 471$ | $0 \cdot 529$ | $0 \cdot 200$ | $0 \cdot 500$ | $0 \cdot 300$ |

| | Male Gametes | | | Female Gametes | |
|---|---|---|---|---|---|
| | $SR$ | $ST$ | $Y$ | $SR$ | $ST$ |
| Selection between gametes at meiosis in heterozygotes | 1 | $0 \cdot 100$ | $0 \cdot 100$ | 1 | 1 |
| Gamete selective values at fertilization | 1 | 1 | 1 | 1 | 1 |

The selective values used as specifications for the programme are shown in Table 2. The overall selective values are the maximum values at 25°C given by Wallace and are used in the programme as zygote selective values with no differential selection at the other stages. For both the overall and specific values, the expected population size was taken as 4000 and the initial population was as in the experimental population (cage No. 11) viz. 500 of each of the genotypes $SR$ and $ST$ males and $SR/SR$ and $SR/ST$ females. Four replicate runs were done with each set of selective values. The results are shown in Figures 1 and 2. Generation length in the experimental population was assumed to be 25 days (Wallace 1948; Dobzhansky and Pavlovsky 1953). Use of specific and overall selective values allows a comparison of the effectiveness of the simulation with each, as well as comparison of experimental and simulated populations.

Figure 1 shows striking differences between the experimental and simulated populations. Sex ratio had been eliminated from the experimental population by generation 9, while in the simulated populations, the frequency of sex ratio is tending to an equilibrium. From the given overall selective values, the expected curve
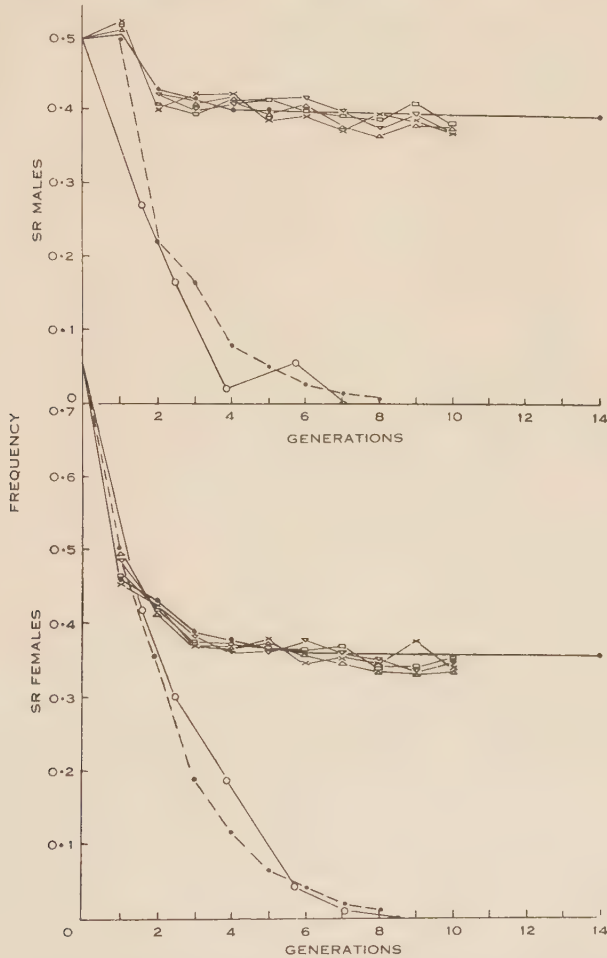


Fig. 1.—Results of competition between sex ratio (*SR*) and standard (*ST*) *X*-chromosomes in *D. pseudoobscura* at 25°C. Maximum overall selective values used in the simulated populations. ○ Wallace's experimental population (cage No. 11). ×, □, △, ▽ Simulated populations. ● —● Calculated theoretical curve for overall selective values of Wallace. ● – – ● Calculated theoretical curve for selective values of 0·95 in *SR* and 1 in *ST* males, and 0 in *SR/SR*, 0·4 in *SR/ST*, and 1 in *ST/ST* females.

of frequency change has been calculated for the first six generations. Bennett (1957) presented formulae which, for given selective values, allow determination of whether an equilibrium is expected, and if so, what will be the equilibrium gene frequencies. If the maximum overall selective values at 25°C are used in these formulae, the equilibrium gene frequencies of sex ratio are 0·393 in males and 0·356 in females.

These values are shown in Figures 1 and 2. The simulated populations in Figure 1 agree closely with one another and fluctuate at random around the expected curve. That is, the simulation provides, for given selective values, an accurate description of the expected changes in frequency in a large population.

The simulated populations using overall selective values (Fig. 1) may be compared with those using specific selective values (Fig. 2). Identity of the curves would not be expected as different sets of random numbers are used in the simulation of chance effects. The curves for males are, however, fairly similar though those in Figure 2 do have a slight excess of values lower than the expected curve.
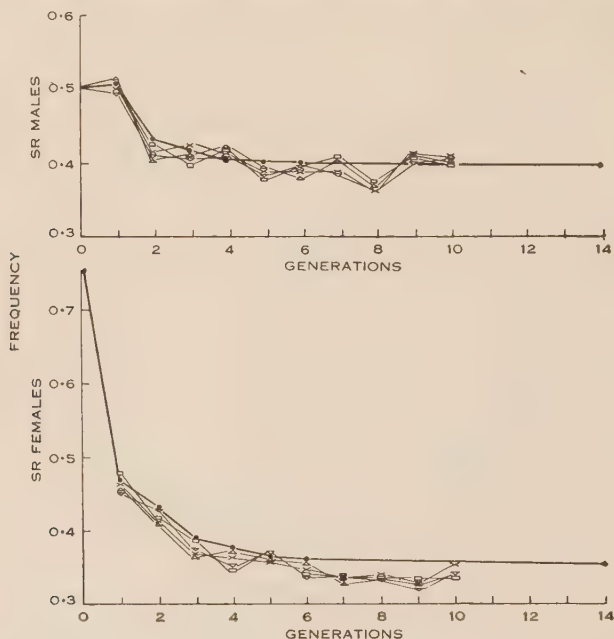


Fig. 2.—Results of competition between sex ratio ($SR$) and standard ($ST$) $X$-chromosomes in *D. pseudoobscura* at 25°C. Maximum specific selective values used in the simulated populations. Designation of populations as in Figure 1.

This could be a chance effect but in females the curves from specific selective values are generally below the expected curve, the difference averaging about 0·02–0·03 in the gene frequency. This could result from the selective values taken for selection between gametes at meiosis in males. The values of 1·0 for $SR$ and 0·1 for $Y$ in $SR/Y$ males will give a bias in favour of $Y$; that is, a possible reduction in the production of $SR$-containing gametes from these males each generation. This bias may be somewhat more than is apparent from the earlier discussion based on Darlington and Dobzhansky's (1942) finding that males comprise 6·2 per cent. of the offspring of sex ratio males. This percentage of male offspring varies for different sex ratio males, and Wallace states that the sex ratio chromosomes used in the experimental population came from 16 males which gave offspring consisting of 0–5 per cent. males. Although the bias would be small, it could build up over a

few generations and its effects would, of course, be more noticeable in females than in males. It is reasonable to conclude that the methods of simulation of selection used are adequate and that specific or overall selective values may be used to study any given genetic situation.

The differences between the results of the experimental and simulated populations emphasize the inadequacy of available methods of estimating fitnesses of competing genotypes. An attempt has been made to determine fitnesses that would fit the experimental population. The calculated curve for the following values

| Males | | Females | |
|---|---|---|---|
| $SR$ | 0·95 | $SR/SR$ | 0 |
| $ST$ | 1 | $SR/ST$ | 0·4 |
| | | $ST/ST$ | 1 |

is shown in Figure 1.

The calculated curve is sufficiently similar to the experimental that these fitness values may be taken as approximating the true fitnesses. They are quite different to the fitnesses (overall selective values in Table 2) experimentally determined by Wallace. The factors of larval competition, longevity, etc., that Wallace studied are clearly important components of fitness, but the fitnesses calculated from them do not fit the observed elimination of sex ratio in the experimental population. Both maximum and minimum estimates of the fitnesses are given by Wallace while only the maximum have been used in the simulated populations. However, even the minimum estimates would not give a selection curve similar to the experimental population as these values would result in an equilibrium frequency of sex ratio in males of 0·405 and in females of 0·363. Wallace states that the factors he studied are obviously not all the components of fitness and mentions various others that could be of importance. These other components may be responsible for the difference between the true fitnesses and the estimated ones. On the other hand, the difference may also be due to errors in estimation. These could result from sampling errors in that generally only small numbers of flies were taken from the cage to start the various estimates. In addition, the estimation procedures may cause errors. For example, the components studied (except for larval competition) cannot be measured under conditions that approach those of a population cage. The relative fitnesses in a cage could be quite different to those as measured in vials. For example, the estimated zygote selection coefficient for $SR/SR$ females of 0·036 could have a true value of zero under cage conditions. It is difficult to see to what extent cage conditions would alter the other components.

To attempt to estimate fitness by studying its components separately will usually give doubtful results because all the components are not necessarily understood, and many whose effects are realized are very difficult to measure accurately. A major requirement is some simple method of estimating overall fitness of different genotypes. Knight and Robertson (1957) have taken one step in this direction by devising a technique for *Drosophila* which involves competition of the genotype under test with a stock marked with two dominant genes (Curly and Plum) and the final assessment is called "the competitive index". Further, the programmes developed here are being used to generate families of selection curves for a wide range of

selective values. Therefore, from the results of experimental populations involving competition between genotypes, it will be possible to obtain estimates of the relative selective values (fitnesses) of the competing genotypes by comparison against the selection curves for series of simulated populations.

### (b) Small Population Size

Merrell and Underhill (1956) analysed competition between the sex-linked mutant *yellow* (*y*) of *D. melanogaster* and its wild-type allele. The populations were maintained in population bottles (Reed and Reed 1948). The relative viabilities of the genotypes and the degree of selective mating were determined. These results can be converted to selective values appropriate for use in the programme. The data given for relative viability of mutant and wild type sibs is:

|         | Numbers of Sib Progeny | |
|---------|----------------|----------------|
|         | + or +/*y*     | *y* or *y*/*y* |
| Males   | 444            | 477            |
| Females | 475            | 440            |

and, from this, zygote selective values have been taken as

| Males | | Females | |
|-------|---|---------|-------|
| +     | 1 | +/+     | 1     |
| *y*   | 1 | +/*y*   | 1     |
|       |   | *y*/*y* | 0·926 |

Table 3 gives results of selective mating experiments (Merrell 1949). The male choice experiments show that wild-type males mated at random with either female type, while the *yellow* males mated significantly more often with the *y*/*y* females than with the +/*y* females. However, where the females have a choice of males (female-choice experiment) the majority of females of both genotypes mate with the wild-type male. There is no way of combining the data from these two experiments (except subjectively) to determine the degree of selective mating when all four genotypes are present. However, in this case the preference of *y*/*y* females for + males will probably greatly exceed that of *y* males for *y*/*y* females. +/*y* and +/+ females will be expected to have the same genotypic reproductive coefficients and it appears simplest to give all three female genotypes equal coefficients, with the selective mating operating only through the male reproductive coefficients. The relative sexual activity of *y* males to + males with *y*/*y* females is 1 : 9 approximately. However, *y* males, being more distasteful to + females, are twice as likely to fertilize *yellow* females as +, so that with + females their relative activity is 1 : 19. In setting up programme specifications, only one overall comparison of + to *y* males is made so that an average estimate must be used. The genotypic reproductive coefficients have been taken as:

| Males | | Females | |
|-------|------|---------|------|
| +     | 0·92 | +/+     | 0·33 |
| *y*   | 0·08 | +/*y*   | 0·33 |
|       |      | *y*/*y* | 0·33 |

It must be emphasized that these values are subjective and approximate. True

estimates could only be obtained from multiple choice experiments on selective mating.

There is no information on differential selection at the gamete stage so that these coefficients were all taken as 1. $E$ was specified as 180. The initial population was taken as 1 wild-type male, 99 *yellow* males, 100 *yellow* females, which correspond closely to the experimental populations where 1 wild-type male was added to an established *yellow* population estimated to contain about 200 individuals. Ten replicate runs were done using these specifications. Eight of these simulated populations became homozygous for $y$, the wild-type allele being lost from four of them

TABLE 3

RESULTS OF SELECTIVE MATING EXPERIMENTS BETWEEN YELLOW AND WILD TYPE

Results quoted from Merrell (1949). Tests carried out with freshly emerged flies except where indicated

| Female-choice Experiment | | | Male-choice Experiment | | |
|---|---|---|---|---|---|
| Female Genotype | Successful Males (%) | | Male Genotype | Fertilized Females (%) | |
| | $y$ | $+$ | | $y/y$ | $+/y$ |
| $y/y$ | 10·5 | 89·5 | $y$ | 69·8 | 30·2 |
| $y/y^*$ | 11·8 | 88·2 | $y^*$ | 72·0 | 28·0 |
| $+/y$ | 4·6 | 95·4 | $+$ | 56·1 | 43·9 |
| $+/y^*$ | 7·8 | 92·2 | $+^*$ | 49·4 | 50·6 |

\* Males and females aged 7 days.

in generation 1, from three in generation 2, and from the other in generation 3. In the remaining two populations, the frequency of $y$ decreased until the populations became homozygous for $+$. This is shown in Figure 3 which compares the results of the experimental and simulated populations. Generation length in the experimental populations was taken as 24 days (Merrell 1953*b*).

Merrell and Underhill (1956) started nine populations of competition between *yellow* and wild-type allele, but the wild-type became established in only three populations (see Fig. 3). Unfortunately, they did not state how long this allele remained in the other six populations before it was eliminated. However, the simulated and experimental populations show close agreement in this as, in eight of the 10 simulated, the wild-type allele was eliminated. The remaining simulated and experimental populations show similar trends in the elimination of *yellow* (Fig. 3).

In this case, the selective values estimated by Merrell and Underhill are sufficient to explain the observed gene frequency changes. However, Morpurgo and Nicoletti (1955, 1956) have queried the importance of selective mating as a factor in gene frequency changes in experimental populations. Their doubts are based on results with *white* and its wild-type allele only, so that they do not necessarily apply to other mutants. Perhaps, as they suggest, different selective values would give similar results. However, as the independently calculated selective values

give results in the simulated populations very similar to the experimental ones, they probably are essentially correct. Selective mating is thus a factor of major importance in this competition. A further argument can be deduced from the early generations of competition. After a varying number of generations where the frequency of *yellow* males remains near 100 per cent., this percentage suddenly decreases. In one generation then, there is a large production of $+$ males, which can only be progeny of $+/y$ or $+/+$ females. If, by chance, the number of $+/y$ females increases in one generation, they will preferentially mate with $+$ males. This will cause an increase in the number of $+/y$ and $+/+$ females in the following generation, and an increase in the number of $+$ males in the next. Morpurgo and Nicoletti's argument is that differential viability in the larval stage is more important than
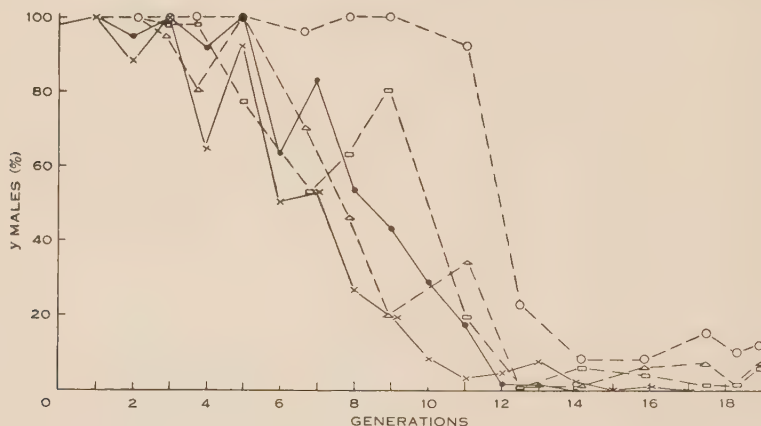


Fig. 3.—Results of competition between $y$ and its wild-type allele in *D. melanogaster*. $\bigcirc$, $\square$, $\triangle$ Experimental populations. $\bullet$, $\times$ Simulated populations.

Merrell and Underhill would consider it. In these early generations, the numbers of $+/+$ and $+/y$ eggs produced will be still quite small relative to the numbers of $y/y$. It is difficult to see then how increased viability of the former relative to the latter could give a sudden increase in one generation in the number of $+/+$ and $+/y$ females.

Comparisons of Figures 1 and 3 shows a greater degree of variation between simulated populations at the smaller population size. Variation in the large population (Fig. 1) is low but cannot be compared with experimental population variation as there is only one such population. In the small populations (Fig. 3) the experimental populations show greater variation than the simulated but with so few populations, this difference may not be real. The whole problem of the interrelation of selective values and population size with variance of the selection curves is being investigated in detail.

In general, the results show that it is possible to simulate in an automatic digital computer the operations of selection between two alleles at a sex-linked locus. This programme is being used in further studies of such selection.

## IV. Acknowledgments

## V. References

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